# Interleukin-10 promoter Single Nucleotide Polymorphism in Non-Hodgkin's Lymphoma and Diffuse Large B-cell Lymphoma

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# Abstract

**Background:**

Interleukin-10 (IL-10) is an important immuno-modulator of the B-lymphocytes. Single Nucleotide Polymorphism (SNP) at the IL-10 promoter positions -597, -824 and -1087 are related to the transcription of the gene and, hence, the serum level of IL-10. The SNP at the IL-10 promoters may, therefore, influence the clinical behaviors of lymphomas. Diffuse Large B-cell Lymphoma (DLBCL) is the most common type of non-Hodgkin's Lymphoma (NHL). The role of SNP in the IL-10 promoter and the prognosis of DLBCL are controversial. Recently, the prognosis of DLBCL is also shown to be related to specific subtypes, namely "germinal center", "activated germinal center" and "activated non-germinal center", according to the expressions of different antigens. The prognostic values of the SNP of the IL-10 promoter and DLBCL subtypes in the local population are also not fully understood.

**Objective:**

The SNP of the IL-10 promoter in different subtypes of primary DLBCL were examined.

**Materials and methods:**

SNP genotyping of the IL10 promoter was performed by Restriction Fragment Length Polymorphism on the genomic DNA extracted from formalin-fixed, paraffin-embedded archival sections of 144 NHL (1987-2004) and 108 primary DLBCL (1995-2005) patients. Genomic DNA from buffy coats of 139 healthy blood donors was used as control. Subtyping of DLBCL was performed with immunohistochemical studies using antibodies for CD10, Bcl-6, CD138 and MUM1. Clinical data were retrieved retrospectively from the case records.

**Results:**

In the local Chinese population, the most common alleles at the IL-10 promoter position -597, -824 and -1087 were A, T and A, respectively. The most common genotype was homozygosity of AA, TT and AA, respectively. A novel linkage of the genotype of SNP of the IL-10 promoter was noted at positions -597 and -824. The SNP profiles of the IL-10 promoter in the local population were those known to be associated with low serum IL-10 background.

The most frequent subtype of DLBCL in the present series was "activated non-germinal center type", in contrast to the "germinal center type DLBCL" in published series, there were no significant correlation on the overall survival of local

DLBCL patients with all the IL-10 promoter SNPs and the different DLBCL subtypes.

In DLBCL patients, TC at -824 position is the most frequent genotype, instead of TT in the controls. Unlike normal controls, the linkage between -597 and -824 was disrupted in some DLBCL patients. As in the normal control, ATA is the most frequent haplotype in DLBCL. However, in DLBCL patients, rare haplotypes were identified, namely ACA, ATC and GCA. These haplotypes were not found in normal controls. Among these three rare haplotypes, ACA was significantly more frequent in DLBCL than in the normal controls.

**Conclusions:**

The background SNP profiles in IL-10 promoter of the local Chinese population, including DLBCL patients, were those associated with low IL-10 production. Unlike published data on the Caucasian population, the spectrum of subtypes of local DLBCL was distinct. The known biomarkers for overall survival of DLBCL, including the SNP of IL-10 promoter and the DLBCL subtyping, appeared less useful clinically in the local settings. The T-allele at the IL-10 promoter position -824 and the rare haplotype ACA may be associated with DLBCL in the local population.

These results suggested ethnical variations in the pathogenesis of DLBCL,

which may be related to a low IL-10 microenvironment. The difference in spectrum of subtypes and variations in prognostic factors of DLBCL not only further highlighted these ethnical differences, but also conferring significant clinical implications in the management of the local DLBCL patients.

# 摘要

**背景:**

白細胞介素-10 是 B 淋巴細胞的重要免疫調節遞質。位於白細胞介素-10 啓動區-597, -824 and -1087 位點的單基因多態性與基因的表達有關，而改變白細胞介素的血清學水平。因此位於白細胞介素-10 啓動區-597, -824 and -1087 位點的單基因多態性可能影響淋巴瘤的臨床表現。彌漫性大 B 淋巴細胞性淋巴瘤是非何傑金氏淋巴瘤的最常見類型。白細胞介素-10 啓動區的單基因多態性與彌漫性大 B 淋巴細胞性淋巴瘤預後的關係尚有爭議。根據個別特定蛋白質不同的表達，彌漫性大 B 淋巴細胞性淋巴瘤可分爲生髮中心型、活化的生髮中心型和活化的非生髮中心型。近來有資料顯示彌漫性大 B 淋巴細胞性淋巴瘤的預後可能與這些亞型有關。對於香港地區的漢族人群來說，白細胞介素-10 啓動區的單基因多態性及彌漫性大 B 淋巴細胞性淋巴瘤的不同亞型對預後的影響尚不清楚。

**目的:**

研究不同亞型的原發性彌漫性大 B 淋巴細胞性淋巴瘤的白細胞介素-10 啓動區的單基因多態性。

**材料和方法:**

基因組 DNA 從 144 例非何傑金氏淋巴瘤(1987 年-2004 年)和 108 例原發性彌漫性大 B 淋巴細胞性淋巴瘤患者(1995 年-2005 年)的蠟塊中提取，用核酸限制性片段長度多態性（RFLP）的方法分析測定白細胞介素-10 啓動區的單基因多態性，對照組 DNA 從 139 例健康志願者中提取。根據彌漫性大 B 淋巴細胞性淋巴瘤的四個生化標記物（CD10, Bcl-6, CD138 和 MUM1）將該組患者進行分型。回顧性查閱患者的病案資料獲得所需的臨床資料。

**結果:**

香港地區漢族人群白細胞介素-10 啓動區-597, -824 and -1087 位點的最常見等位基因分別是 A-allele, T-allele 和 A-allele，最常見的基因型分別是純合子 AA, TT 和 AA 。在白細胞介素-10 啓動區-597 和 -824 位點發現一個新的單基因多態性的基因型連接。白細胞介素-10 啓動區的單基因多態性圖譜與當地人群的白細胞介素-10 的低血清學水平有關。

本研究人群中最常見的彌漫性大 B 淋巴細胞性淋巴瘤的亞型是活化的非生髮中心型，不同於文獻報導的生髮中心型。白細胞介素-10 的單基因多態性及彌漫性大 B 淋巴細胞性淋巴瘤的亞型對患者的整體生存均沒有影響。

彌漫性大 B 淋巴細胞性淋巴瘤患者-824 位點最常見的基因型是 TC，而對照組中 TT 最爲常見。不同于正常對照組，彌漫性大 B 淋巴細胞性淋巴瘤患者-597

和 -824 位點的基因型並未有如正常對照組一樣具連接性。彌漫性大 B 淋巴細胞性淋巴瘤最常見的單倍體是 ATA，這一點與正常對照組相同。然而，在彌漫性大 B 淋巴細胞性淋巴瘤也發現一些較少見的單倍體，如 ACA, ATC 和 GCA，正常對照組中則沒有這些單倍體。在這三種少見的單倍體中，ACA 在彌漫性大 B 淋巴細胞性淋巴瘤患者中的發生率明顯高於正常對照組。

**結論:**

香港地區漢族人群（包括彌漫性大 B 淋巴細胞性淋巴瘤患者）白細胞介素-10 啓動區的單基因多態性圖譜與低血清白細胞介素-10 水平有關。本人群彌漫性大 B 淋巴細胞性淋巴瘤的亞型分佈特點不同于西方人群。雖然文獻報導白細胞介素-10 啓動區的單基因多態性和免疫生化亞型是影響彌漫性大 B 淋巴細胞性淋巴瘤整體生存的生化指標，但是這些指標在本研究人群中的臨床意義不大。白細胞介素-10 啓動區-824 位點的 T-allele 和少見單倍體 ACA 可能與當地人群的彌漫性大 B 淋巴細胞性淋巴瘤的臨床特點有關。

以上結果顯示了彌漫性大 B 淋巴細胞性淋巴瘤發病機制的種族特異性，這種特異性可能與當地人群低血清白細胞介素-10 的微環境有關。本研究發現彌漫性大 B 淋巴細胞性淋巴瘤亞型分佈和預後影響因數均與西方人群不同，這個發現的不同不僅提示了該病的種族特異性，而且對治療方面有重要的臨床意義。

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# List of Abbreviations

| | |
|---|---|
| AGC | Activated germinal center |
| ANGC | Activated non germinal center |
| BSA | Bovine serum albumin |
| BTB | Bric-a-brac, Tramtrack, Broad complex |
| CALLA | Common Acute Lymphoblastic Leukemia Antigen |
| CDK | Cyclin-Dependent Kinases |
| cDNA | Complementary DNA |
| CLL | Chronic Lymphocytic Leukemia |
| CNS | Central Nervous System |
| CRF | Cytokine receptor family |
| CSIF | Cytokine Synthesis Inhibitory Factor |
| DAB | Diaminobenzidene |
| DLBCL | Diffuse Large B-cell Lymphoma |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein Barr Virus |
| ELISA | Enzyme Linked Immunosorbent Assay |
| Ets | Epithilium specific transcription factor |
| GC | Germinal center |
| HD | Hodgkin Disease |
| HLA | Human Leukocyte Antigens |
| HRP | Horseradish peroxidase |
| IFNR | Interferon receptor |
| IκB | I-Kappa B |
| IKK | I-Kappa B Kinase |
| IL-10 | Interleukin 10 |
| IL-10R | Interleukin 10 recpetor |
| IPI | International Prognostic Indicator |
| JAK | Janus kinase |
| LD | Linkage Disequilibrium |
| mRNA | Messanger RNA |
| NFκB | Nuclear Factor Kappa light polypeptide gene enhancer in B-cells |
| NHL | Non Hodgkin's Lymphoma |
| NK | Natural Killer |
| OS | Overall Survival |

| | |
|---|---|
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| POZ | Pox virus zinc finger |
| Rb | Retinoblastoma |
| RNA | Ribonucleic acid |
| RS | Reed-Sternberg |
| SDS | Sodium dodecyl sulphate |
| sIg | Surface Immunoglobulin |
| SLL | Small Lymphocytic Leukemia |
| SNP | Single Nucleotide Polymorphism |
| STAT | Signal transducers and activators of transcription |
| TBS | Tris buffered saline |
| Th | T helper |
| Tyk | Tyrosine kinase |
| UC | Unclassified |
| WHO | World Health Organization |

# Chapter 1: Introduction

## 1.1 Malignant Lymphoma

Malignant lymphomas are a group of tumors derived from cells of the lymphoreticular system. Lymphomas are divided into two broad categories, Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL), depending on the histopathological identification of the type of neoplastic cell (Burkitt et al, 1996). HD is defined morphologically by the presence of scattered diagnostic neoplastic Hodgkin's and Reed-Sternberg (RS) cells in a background of non-neoplastic inflammatory and accessory cells. NHL comprises the remaining forms of lymphoma besides HD. Globally, there were 62,329 HD cases in the year 2002, comprising about 17.18 % of all lymphoma cases. The mortality rate was 36.60%. The female to male ratio was 1:1.6 (IARC Globocan 2002 website). In Asia, HD is relatively rare which comprised only 9.2% in malignant lymphoma in a study of 294 adult Chinese lymphoma patients (Shih et al, 1991).

## 1.2 Non-Hodgkin's Lymphoma

NHL is a large heterogeneous group of disorder characterized by malignant proliferation of lymphoid cells (Burkitt et al, 1996). The cellular origins of most

types are increasingly well established with subtypes corresponding to the various stages of lymphocyte differentiation. Depending on the respective immunophenotypes, NHL can be sub-grouped into B, T and NK-cell lymphomas (Kato et al, 1999).

There were 300,571 NHL cases in 2002 worldwide with a mortality rate of 57.16%. The female to male ratio was 1:1.4. In China, there were 30,440 NHL cases in 2002 with comparable mortality rate of 60.47%. The female to male ratio was 1:1.94 (IARC Globocan 2002 website).

While HD is typically localized to lymph nodes (Swedlow, 2003), NHL can arise in both nodal and extranodal sites. NHL affects not only major lymphoid tissue-bearing sites such as the tonsil (Levine et al, 1985), it can also involve locations devoid of native lymphoid tissue, such as the skin (Isaacson, 1994). Of particular relevance to the local situation is that extranodal NHL has relatively higher incidence in Asia (Ho et al, 1984; Harris et al, 1994).

## 1.3 Diffuse Large B-cell Lymphoma

### 1.3.1 General Features of Diffuse Large B-cell Lymphoma

Diffuse Large B-cell Lymphoma (DLBCL) is a diffuse proliferation of large neoplastic B lymphoid cells with nuclear size exceeding normal macrophage nuclei

or more than twice the size of a normal lymphocyte (Jaffe et al, 2001). It was reported to be the most common type of lymphoma as defined in the Revised European-American Lymphoma (REAL) classification, representing 40% of the new cases of lymphomas diagnosed each year (Coiffier, 2001). The median age of DLBCL is in the 7$^{th}$ decade, but the range is broad, and it is slightly more common in males than females (Jaffe et al, 2001). This trend of DLBCL remains the same under the new classification of the World Health Organization.

DLBCL may present in nodal or extra-nodal sites. Up to 40% are confined to extra-nodal sites and the most common extra-nodal site is the gastrointestinal tract (stomach or ileo-cecal region) (Harris et al, 1994). However, DLBCL can virtually present in any extra-nodal location including the skin, the central nervous system (CNS), the bone, the testis, the soft tissue, the salivary glands, the female genital tract, the lungs, the kidneys, the liver, the Waldeyer's ring and the spleen (Jaffe et al, 2001).

The causes of DLBCL remain unknown (Coiffier, 2001). They usually arise *de novo* (primary DLBCL) but can be resulted from progression or transformation (secondary DLBCL) of a less aggressive lymphoma such as chronic lymphocytic leukemia/ small lymphocytic lymphoma (CLL/SLL), follicular lymphoma, marginal zone B-cell lymphoma or nodular lymphocyte predominant Hodgkin's disease. (Jaffe

et al, 2001).

Although most patients respond initially to chemotherapy, only 35 to 40% of patients achieve durable remission (Rosenwald et al, 2003) and more than half succumb to their disease (de Leval et al, 2003).

DLBCL is much more heterogeneous, in terms of clinical behavior and histomorphologic features, than other groups of lymphomas (Brittinger et al, 1984). DLBCL occurs over a broad range of ages and manifests at nodal or extranodal sites (Harris et al, 1994). DLBCL consists of several morphologic variants including anaplastic, centroblastic, immunoblastic, and T-cell/histiocyte-rich subtype (Reed et al, 1977). However, no distinct prognostic significance has been correlated with any of these histomorphologic variants in recent lymphoma classification systems (Harris et al, 1994).

### 1.3.2 Morphologic variants of Diffuse Large B-cell Lymphoma

DLBCL are composed of large transformed lymphoid cells. Cytologically, DLBCL are very diverse and can be divided into several morphologic variants, namely, centroblastic, immunoblastic and anaplastic (Harris et al, 1994). However, distinction among these variants has generally met with both poor intra-observer and inter-observer reproducibility (Harris et al, 1994). Moreover, prognosis of patients

cannot be predicted by this classification. Immunopehnotypic and genotypic parameters are suggested to provide superior prognostic information of DLBCL patients (Chang et al, 2004; Alizadeh et al, 1999).

### 1.3.2.1 Centroblastic vairant

This is the most common morphologic variant and is composed of medium sized to large lymphoid cells with oval to round, vesicular nuclei containing fine chromatin and 2 to 4 membrane bound nucleoli. The cytoplasm is generally scanty and amphophilic to basophilic. This variant may have a monomorphic or polymorphic appearance (Engelhard et al, 1997). In some cases the cells may be multilobated.

### 1.3.2.2 Immunoblastic variant

More than 90% of cells in this variant are with a single, centrally-located nucleolus and an appreciable amount of basophilic cytoplasm. Immunoblasts with plasmacytoid differentialtion may also be present. Less than 10% of centroblasts are present in the population. (Jaffe et al, 2001)

#### 1.3.2.3 Anaplastic variant

This variant is characterized by very large round, oval or polygonal cells with bizarre pleomorphic nuclei which may resemble Reed-Sternberg cells. The cells may grow in a cohesive pattern, mimicking carcinoma, or may show a sinusoidal pattern of growth (Haralambieva et al, 2000).

### 1.3.3 Immunophenotype of Diffuse Large B-cell Lymphoma

#### 1.3.3.1 Lineage-associated antigens

##### 1.3.3.1.1 B-cell lineage antigens

DLBCL usually expresses CD45 (leucocyte common antigen) and pan-B-cell antigens, such as CD19, CD20 and CD79a, but may lack one or more of these markers (Jaffe et al, 2001). CD20 is a transmembrane protein that functions as a $Ca^{2+}$ permeable cation channel. CD20 is expressed on B-cells from the stage of the precursor B-cell until the preplasma cell stage of differentiation (Chang et al, 1996). CD79a, which is part of a heterodimer associated in the B-cell membrane with immunoglobulin, is expressed on precursor B-cells and is retained throughout B-cell differentiation until a later stage than CD20. In most plasma cells, CD79a is positive (Mason et al, 1995). Both CD20 and CD79a are highly specific markers of B-cell lineage. Rare cases of peripheral T-cell lymphomas were positive for CD20

(Quintanilla-Martinez et al, 1994).

Intrinsic surface immunoglobulin (sIg) is a specific marker for B-cells that is expressed uniquely by this lymphocyte population and serves as the antigen-recognition molecule for B-cells (Knowles, 2001). The majority of B-cell lymphomas, including DLBCL, express monotypic sIg, usually of the IgM subtype (Lamarre et al, 1989). Since the Ig idiotype is a potential target of cytotoxic cells (Kwak et al, 1992), defective expression of the sIg might be a route for immune escape.

**1.3.3.1.2 T-cell lineage antigens**

CD5, found primarily in T-cells, is also expressed by a small subset of normal B-cells, the B-1 cells (Kipps, 1989). CD5 is a characteristic antigen of CLL/SLL and mantle cell lymphoma (MCL). Only a small proportion of about 10% of the DLBCL expresses CD5 (Burns et al, 1983). CD5 positive DLBCL occurs *de novo* rather than as a manifestation of transformation of CLL/SLL (Yamaguchi et al, 2002). CD5 positive DLBCLs are negative for cyclin D1 expression, distinguishing them from blastoid MCL (Jaffe et al, 2001). The de novo CD5-positive DLBCL is a highly aggressive subtype of DLBCL. CD5–positive DLBCL has significantly poorer prognosis than those of CD5 negative cases (Yamaguchi et al, 2002).

#### 1.3.3.2 Antigen involved in regulation of cell proliferation and apoptosis

##### 1.3.3.2.1 Proliferation markers

Several studies have examined the impact of cell proliferation in DLBCL. The monoclonal anti-Ki67 antibody is widely used as a proliferation marker (Gerdes et all, 1984). The proportion of cells expressing Ki67 defines the fraction of actively cycling cells. In DLBCL, the cell cycle fraction is variable, usually ranging from 30 to 100%, but is typically high with median values around 65% (Hall et al, 1988). Moreover, it has been reported that high expression of Ki67 in DLBCL patients was associated with shorter overall survival and, hence, with poorer prognoses (Sanchez et al, 1998).

##### 1.3.3.2.2 Cell cycle regulators

Cell cycle progression is regulated by a complex molecular network involving cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors. The two major regulatory pathways are the p53 pathway, which regulates apoptosis and cell cycle arrest in G1, and the Rb pathway, which regulates the transition from G1 to S. Genetic alterations and/or dysregulation of these factors are frequently detected in DLBCL.

The *p53* gene is one of the most frequently mutated genes in human cancer. The

p53 protein, referred to as 'the guardian of the genome', monitors DNA integrity. The p53 protein arrests cells at G1 or programs the cells to cell death when DNA replication is defective or when DNA is damaged (Levine et al, 1991). Mutations of the *p53* gene, leading to a functionally defective protein, have been detected in about 20% of DLBCL (Ichikawa et al, 1997). Mutations of the *p53* gene in DLBCL are associated with clinical drug resistance (Wilson et al, 1997) and poor outcome (Leroy et al, 2002). The p53 protein is not detected by immunohistochemistry in normal cells because of its rapid degradation. Either *p53* gene mutation affecting p53 turnover, or accumulation of wild-type p53 can result in detectable protein. In DLBCL, p53 is detectable by immunohistochemistry in 30 to 40% of the cases. In some of these cases, immunoreactivity of p53 in DLBCL cells is seen in the absence of mutation of the *p53* gene (Gascoyne, 1997).

The retinoblastoma (Rb) protein negatively regulates the G1/S transition of the cell cycle (Sanchez et al, 1998). In DLBCL, loss of Rb expression is an adverse prognostic factor. High Rb expression (>80% positive cells) is associated with extended survival times (Sanchez et al, 1998).

#### 1.3.3.2.3 Protein controlling apoptosis

The Bcl-2 protein is located in the mitochondrial inner membrane functions and acts as an anti-apoptotic protein, protecting the cells from programmed cell death (Hockenbery et al, 1990). In DLBCL, Bcl-2 protein expression is found in 30 to 60% of the cases. Bcl-2 immunoreactivity is more frequently detected in nodal form than in extranodal DLBCL (Piris et al, 1994). Expression of Bcl-2 protein is related to poor prognosis in DLBCL patients (Biasoli et al, 2005). With a combination of poor prognostic factors, DLBCL patients of high International Prognostic Indicator, having Bcl-2 protein expression and the lack of germinal center phenotype are 100% fatal in less than 3 years (Barrans et al, 2002).

### 1.3.4 Subtypes of Diffuse Large B-cell Lymphoma

It has been well documented that DLBCL expressing germinal center phenotype-related protein, Bcl-6, is associated with better prognosis (Barrans et al, 2002; Lossos et al, 2000). Recent studies substantiated this finding and refined the classification of DLBCL, with the employment of new technologies including DNA microarray technique (Alizadeh et al, 2000) and immunohistochemical technique with a panel of antibodies (Chang et al, 2004). It is important to develop a technique that can classify the subtype of DLBCL accurately for the prognostic estimation.

### 1.3.4.1 Classification method of DLBCL subtypes

#### 1.3.4.1.1 DNA microarray

Technical and analytical advances make it possible to investigate the expression of thousands of genes at the same time using complementary DNA microarray. To apply this technique to research in normal and malignant lymphocytes, a 'Lymphochip', which contains genes that are preferentially expressed in lymphoid cells and genes with known or suspected role in the processes important in immunology or cancer, was developed (Alizadeh et al, 2000). This Lymphochip contains 12,069 cDNA clones chosen from a germinal center B-cell library, and 2,338 cDNA clones from libraries derived from DLBCL, follicular lymphoma, mantle cell lymphoma and chronic lymphocytic leukemia libraries. In addition, clones representing a variety of genes that are induced or repressed during B- and T-lymphocyte activation by mitogens or cytokines and a curated set of 3,186 genes of importance to lymphocyte and/or cancer biology were also added to this Lymphochip (Alizadeh et al, 1999).

Clustering analyses of the patterns of gene expressions in DLBCL identified two large branches of DLBCL, namely, the germinal center B-cell DLBCL and the activated B-cell DLBCL. The germinal center B-cell DLBCL expresses, to a varying degree, all of the genes that define the germinal center B-cell signature. In contrast,

the activated B-cell DLBCL expresses these genes at low or even undetectable levels, Fig. 1.1a. A distinct set of genes, corresponding to activation of the B-cells, are differentially expressed in this second large subgroup of DLBCL, Fig. 1.1c. The germinal center B-cell DLBCL and activated B-cell DLBCL could not be distinguished morphologically. More importantly, there is association of germinal center B-cell DLBCL with better prognosis. Hence, DNA microarry analysis provides a way to molecular classify DLBCL by distinguishing germinal center B-cell DLBCL and activated B-cell DLBCL with their respective specific gene expression patterns. Such classification is useful and can allow prognostic information to be estimated.

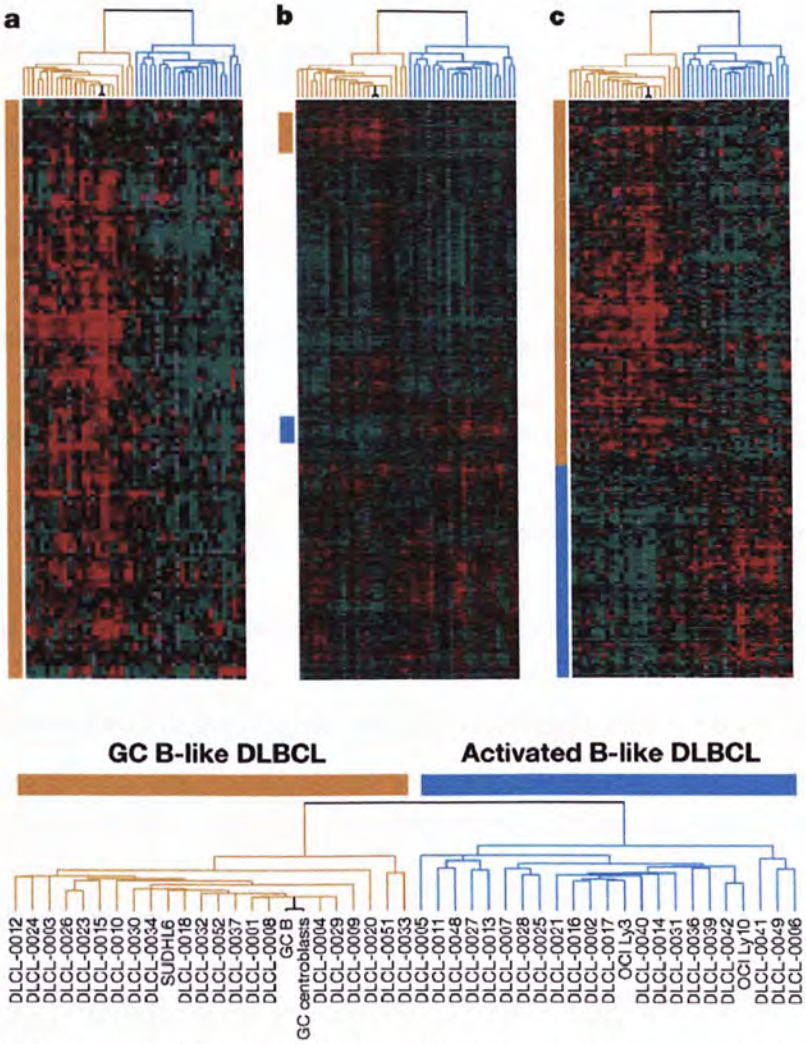



**Figure 1.1** Discovery of DLBCL subtypes by gene expression profiling. The samples used in this clustering analysis are shown as bottom. **a,** Hierarchical clustering of DLBCL cases (blue and orange) and germinal center B-cells (black) based on the genes of the germinal center B-cell gene expression signature. Two DLBCL subgroups, germinal center B-cells DLBCL (orange) and activated B-cell DLBCL (blue) were defined by this process. **b,** Discovery of genes that are selectively expressed in germinal center B-cell DLBCL and activated B-cell DLBCL. All genes from Lymphochip, with the exception of the genes in the proliferation, T-cell and lymph-node gene expression signatures, were ordered by hierarchical clustering while maintaining the order of samples determined in Fig.1a. Genes selectively expressed in germinal center B-cell DLBCL (orange) and activated B-cell DLBCL (blue) are indicated. **c,** Hierarchical clustering of the genes selectively expressed in germinal center B-cell DLBCL and activated B-cell DLBCL, which was determined from Fig. 1b. (Alizadeh et al, 2000)

#### 1.3.4.1.2 Immunohistochemistry pattern

Although thousands of genes of DLBCL can be studied in parallel by DNA microarray, the gene expression profiling studies are currently not practical in the clinical laboratory setting because of the exceedingly high cost associated with the cDNA microarray technology and the need for fresh or frozen tissue.

For routine clinical practice, immunohistochemical markers may be used on formalin-fixed paraffin-embedded tissue to classify DLBCL (Chang et al, 2004). DLBCL subtypes can be classified by staining CD10 and Bcl-6, which are known markers for the germinal center B-cell. These two markers are also shown to be highly expressed in germinal center B-cell DLBCL in DNA microarray experiments. MUM1/IRF4 and CD138, which are expressed by the activated B-cells that have the capacities of differentiation toward plasma cells, can be used in parallel for the identifying of activated B-cells component. With these four antigens, 4 subtypes of DLBCL can be classified, namely, germinal center DLBCL, activated germinal center DLBCL, activated non-germinal center DLBCL and an unclassified category (Chang et al, 2004 and Table 1.1). Fig. 1.2 shows a schematic diagram summarizing the expression of these 4 antigens with respect to various stages of B-cell differentiation.

| | CD10 and/or Bcl-6 | CD138 and/or MUM1 |
|---|---|---|
| **Germinal Center** | +[a] | - |
| **Activated Germinal Center** | + | + |
| **Activated Non-Germinal Center** | - | + |
| **Unclassified** | - | - |

**Table 1.1** Subtyping of DLBCL by immunohistochemical staining of antibodies against CD10, Bcl-6, CD138 and MUM1

[a] positivity is defined as any staining intensity with more than 20% of lymphoma cells showing the specific staining patterns of the respective antibodies.

#### 1.3.4.1.2.1 CD10

CD10 is also known as the Common Acute Lymphoblastic Leukemia Antigen (CALLA). It is a membrane metalloproteinase that is detected in early lymphoid progenitors as well as in a variety of stromal and epithelial cells (LeBien et al, 1989). CD10 is expressed by the majority of follicular lymphomas, while other small B-cell lymphomas do not express this antigen. In DLBCL, CD10 expression on paraffin sections is detected in 20-40% of the cases (de Leval et al, 2003).

#### 1.3.4.1.2.2 Bcl-6

Bcl-6 is a nuclear phosphoprotein belonging to the BTB/POZ (bric-a-brac, tramtrack, broad complex/Pox virus zinc finger) zinc finger family of transcription factors (Zollman et al, 1994). It is an effective transcriptional repressor of wide variety of genes (Chang et al, 1996). Along the B-cell lineage, Bcl-6 is specifically expressed only in mature B-cells within the germinal center (Cattoretti et al, 1995). Bcl-6 is essential for the formation of the germinal center B-cells (Yeh et al, 1997). The mRNA of Bcl-6 and its protein are significantly higher in germinal center B-cell than resting B-cell. However, the exact function of Bcl-6 in germinal center formation of B-cell remains unclear (Allman et al, 1996). Bcl-6 is not found in pre-B-cells or in the differentiated progenies, for example plasma cells. These

observations suggested that Bcl-6 is down regulated in post germinal center B-cells. In DLBCL, about 35 to 40% of cases expressing Bcl-6 (Pasqualucci et al, 2003).

#### 1.3.4.1.2.3 CD138

CD138, also known as Syndecan-1, is an important transmembrane heparin sulphate proteoglycan which is expressed by normal bone marrow-derived B-cell precursors. CD138 is absent in the naive and germinal center B-cells. CD138 is then re-expressed as B cells exit the germinal center and mature in to plasma cells (Sanderson et al, 1989). Little is known about the normal function of CD138 and its role in lymphoid malignancies.

#### 1.3.4.1.2.4 MUM1/IRF4

MUM1/IRF4 protein is the gene product involved in the myeloma-associated chromosomal translocation t(6;14)(p25;q32). MUM1 is a phenotypic marker for B-cell differentiation. In normal B-cells, MUM1 expression is thought to denote the final step of intra-germinal centre B-cell differentiation and subsequent steps of maturation towards plasma cells. In normal lymphoid tissue, MUM1 expression is detected by immunohistochemistry (Falini et al, 2000) with cytoplasmic/nucleus pattern. In DLBCL, MUM1 is detected in 50 to 75% of the cases. Its expression in

DLBCLs may reflect derivation from B-cells at a late germinal center or post-germinal center stage of differentiation, up to the terminal stage of the plasma cell (de Leval et al, 2003).

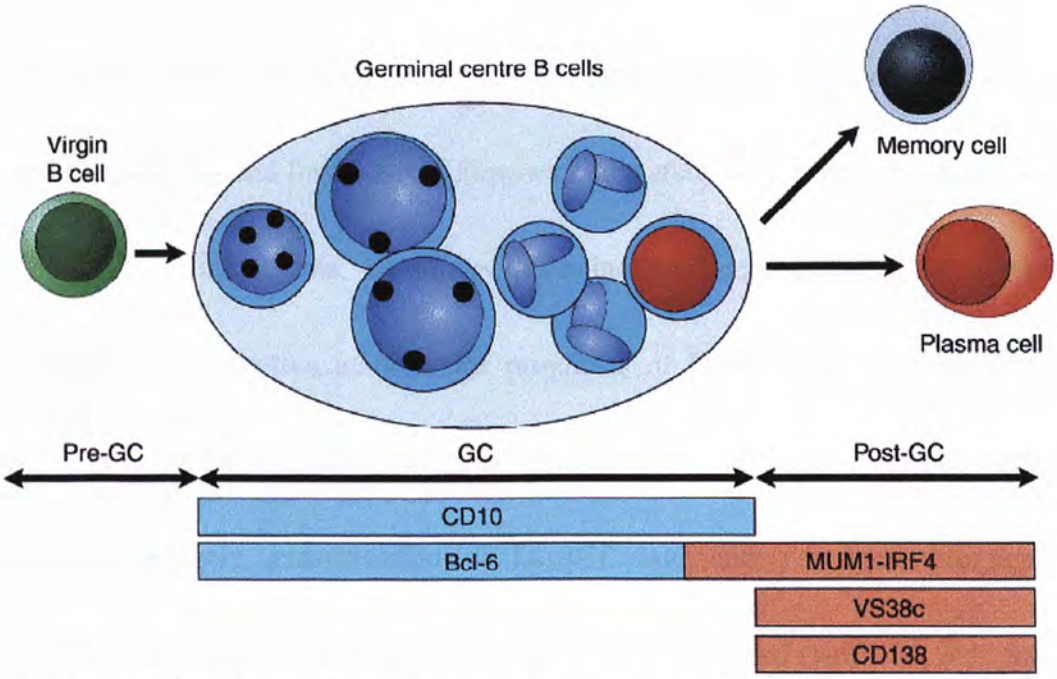



**Figure 1.2** This scheme depicts expression of stage-specific differentiation antigens in relation to normal B-cell differentiation, i.e. pre-germinal center (GC), GC and post-GC stages. (de Leval et al, 2003)

### 1.3.4.2 Prognosis of DLBCL subtypes

Clinical data are being used to provide prognostic information for DLBCL. An International Prognostic Indicator (IPI) is defined taking into account patient's age, performance status, and the extent and location of the disease. The IPI correlates with the prognosis of subtypes in DLBCL. This indicator provides an effective index for therapeutic planning and for research purposes in the study of clinical outcomes.

Besides clinical data, laboratory investigations were explored to provide reproducible and objective markers for prognosis of DLBCL. In both studies of Alizadeh et al. and Chang et al., using DNA microarray and immunohistochemistry method, respectively, classification of DLBCL into subtypes have prognostic significance. Both studies showed that patients in the activated B-cell DLBCL have distinctly worse overall survival than patients in the germinal center B-cell subtype (Alizadeh et al, 2000; Chang et al, 2004). The Kaplan-Meier plot of survival data of patients of both studies are shown in Fig. 1.3 and 1.4, respectively.

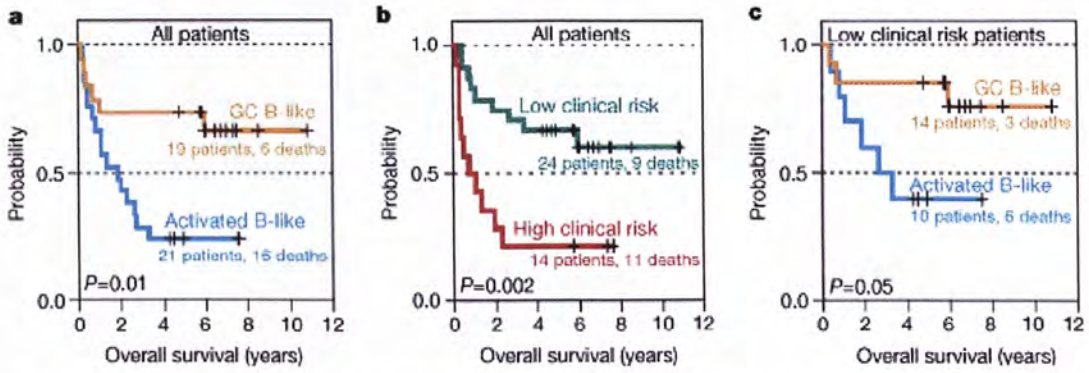



**Figure 1.3** Clinically distinct DLBCL subtypes defined by gene expression profiling. **a,** Kaplan-Meier plot of overall survival of DLBCL patients grouped on the basis of gene expression profiling. **b,** Kaplan-Meier plot of overall survival of DLBCL patients grouped according to the International Prognostic Index (IPI). Low clinical risk patient (IPI score 0-2) and high clinical risk patients (IPI score 3-5) are plotted separately. **c,** Kaplan-Meier plot of overall survival of low clinical risk DLBCL patients grouped on the basis of their gene expression profiles. (Alizadeh et al, 2000)

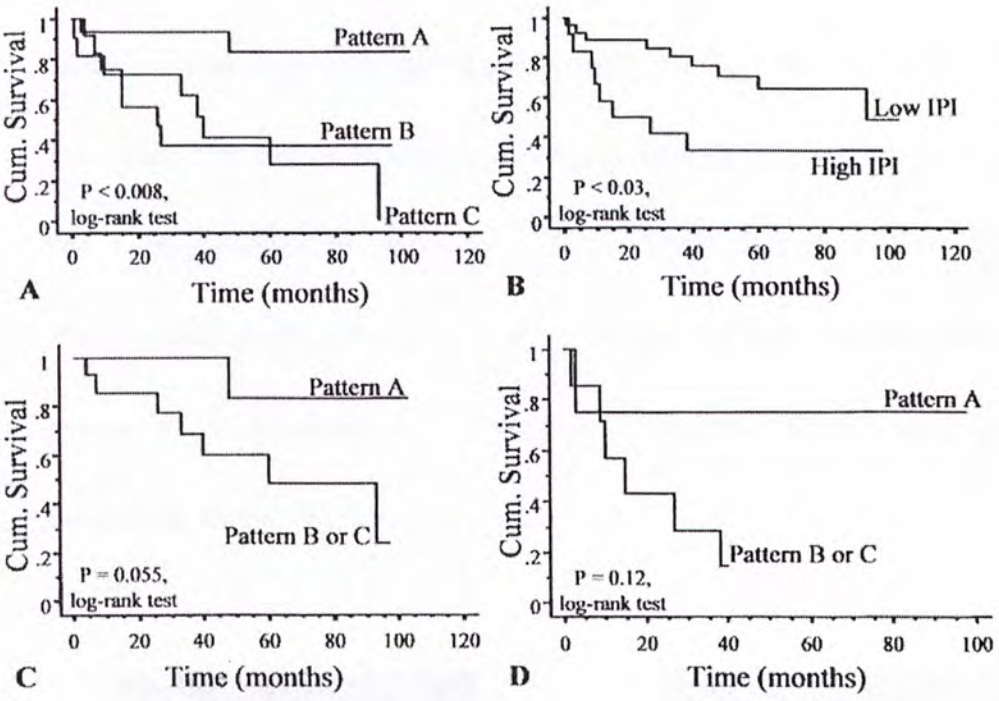
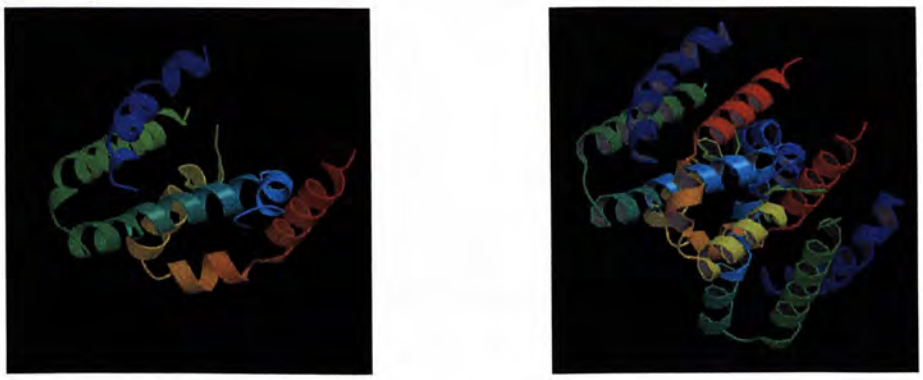



**Figure 1.4 A:** Patients with expression pattern of germinal center B-cell DLBCL have a better overall survival (OS) than those with the other two patterns. **B:** Patients with low IPI scores have a better OS than those with high IPI scores. **C:** Among patients with low IPI score, the patients with expression of pattern of activate germinal center and activated non-germinal center DLBCL have a worse OS than those with expression pattern of germinal center DLBCL. **D:** Among patients with high IPI score, the same trend is observed. (Chang et al, 2004)

## 1.4 Interleukin 10

Interleukin 10 (IL-10) was first described as cytokine synthesis inhibitory factor (CSIF) (Fiorentino et al, 1989) and is activity produced by mouse Th2 cells that inhibit the activation and production of cytokine by Th1 cells. The IL-10 protein is 18kDa in molecular weight and consists of 178 amino acids. Early studies suggested that IL-10 is dimeric (Fiorentino et al, 1989). Biochemical (Syto et al, 1998) and X-ray crystallography (Zdanov et al, 1995) analyses of IL-10 demonstrated that the protein is an acid-sensitive, non-covalent homodimer of two interpenetrating polypeptide chains, Fig. 1.5.



**A** **B**

**Figure 1.5 A,** Monomer of Interleukin 10. **B,** Non-covalent Homodimer of Interleukin 10. (Research Collaboratory for Structural Bioinformatics, Protein Data Base website)

### 1.4.1 The IL-10 gene

The IL-10 gene consists of five exons and is located in chromosome 1 (Spits and de waal Malefyt, 1992). Upon activation, IL-10 gene expresses a 2kb mRNA. IL-10 is expressed by varieties of cells, such as T-cells, monocytes and macrophages (Moore et al, 2001). IL-10 transcription can be regulated by the transcription factors, Sp1 and Sp3. The latter two genes are expressed constitutively by many different cell types (Tone et al, 2000). The stability of IL-10 mRNA can also be controlled at the post-transcriptional level by alteration of the post-transcriptional RNA degradation mechanism (Powell et al, 2000), this further facilitates rapid control of IL-10 expression.

### 1.4.2 IL-10 promoter

The IL-10 promoter is highly polymorphic. Two informative microsatellites, IL-10.G and IL-10.R, are located 1.2kb and 4kb upstream of the transcription start site, respectively (Eskdale et al, 1997). A correlation of microsatellite IL-10.R and low IL-10 secretion of lipopolysacharide (LPS)-induced peripheral blood mononuclear cells in vitro was reported (Eskdale et al, 1998).

There are three frequent point mutations -1087(G/A), -824(C/T), and -597(C/A) (Turner et al 1997; Hurme et al, 1998) in the IL-10 promoter. The -1087

G-allele was associated with higher IL-10 production (Turner et al, 1997). The transcription factors of the Ets family are key regulators of the genes involved in the immune response and cellular proliferation. In the IL-10 promoter, the -1087 A-allele matches the GGAA Ets binding site core motif, but the -1087 G-allele does not. The specific transcription factor for the IL-10 promoter was further characterized as PU.1. PU.1 is an Ets family member expressed in all hematopoetic cell lineages except the peripheral T-cells. It was further demonstrated that the -1087 A-allele, but not the G-allele, confers optimal affinity for PU.1. The binding of the latter inhibits IL-10 expression (Reuss et al, 2002). Therefore, the -1087 G-allele is associated with high IL-10 secretion.

### 1.5 IL-10 receptor

IL-10 receptor is composed of two different chains, the α (Ho et al, 1993) and β (CRFB4) (Kotenko et al, 1997) chains. The β chains belong to the interferon receptor (IFNR) family (Moore et al, 2001).

IL-10Rα, mapped on chromosome 11q23.3, is the ligand-binding subunit that binds IL-10 with high affinity ($K_d$ ~35-200pM) (Liu et al, 1994; Tan et al, 1993). Immunoprecipitation study indicated a molecular size of 90-120kDa for IL-10Rα (Liu et al, 1994). Epitope and peptide mapping data generally supported these

observations (Tan et al, 1995).

IL-10Rβ was originally described as orphan IFNR family member CRFB4/CRF2-4 located in the IFNR gene complex on chromosome 21 (Lutfalla et al, 1993; Gibbs et al, 1997). It is essential for IL-10 mediated effects and CRFB4-deficient mice display the same phenotype as IL-10 deficient mice (Spencer et al, 1998). Its principal function appears to be recruitment of a Jak kinase (Tyk2) into the signaling complex (Kotenko et al, 1997).

## 1.6 Cellular Signaling Pathways Regulated by IL-10

### 1.6.1 Jak/Stat Pathway

The IL-10/IL-10R interaction engages the Jak family tyrosine kinases Jak1, being associated with IL-10Rα, and Tyk2, being associated with IL-10Rβ (Finbloom et al, 1995). IL-10 induces tyrosine phosphorylation and activation the latent transcription factors Stat3, Stat1 and, in non-macrophage cells, Stat5 (Finbloom et al, 1995).

The relationship between the biology of IL-10 and IL-10 signaling molecules and pathways has been identified in both in vitro and in knock out mice studies. Macrophages from Jak1 -/- mice do not respond to IL-10 (Rodig et al, 1998), which indicates that Jak1 plays an obligatory role in IL-10 signaling. Stat3 is also

implicated strongly as a key mediator of IL-10 responses. Stat3 is recruited directly to the IL-10/IL-10R complex via either of the two tyrosine residues in the IL-10Rα cytoplasmic domain. These tyrosine residues are phosphorylated in response to IL-10 (Weber-Nordt et al, 1996) and these tyrosine residues are required for IL-10 signaling (Weber-Nordt et al, 1999). Overexpression of a dominant negative Stat3 mutant or an inducibly active form of Stat3 demonstrated that Stat3 activation was both necessary and sufficient to mediate inhibition of macrophage proliferation by IL-10 (O'Farrell et al, 1998).

In contrast to Stat3, the roles of Stat1 and Stat5 in the IL-10 biology and signal transduction remain unclear. Stat1 and Stat5 do not appear to interact directly with the IL-10/IL-10R complex (Weber-Nordt et al, 1996). Overexpression of dominant negative Stat1 or Stat5 did not block IL-10's effects on a macrophage cell line (O'Farrell et al, 1998). Moreover, macrophages from Stat1 -/- mice remain responsive to IL-10 (Meraz et al, 1996).

### 1.6.2 Inhibition of NFκB pathway

IL-10 controls inflammatory processes by suppressing the expression of pro-inflammatory cytokines, chemokines, adhesion molecules, as well as antigen-presenting and co-stimulatory molecules in monocytes/macrophages,

neutrophils and T-cells. (Moore et al, 2001). As all of these inflammatory proteins are transcriptionally controlled by NFκB, it was suggested that IL-10 may exert a significant part of its anti-inflammatory properties by inhibiting this transcription factor.

IL-10 inhibits NFκB activity in two different ways: 1) IL-10 blocks NFκB nuclear translocation by inhibiting IKK activity (Yin et al, 1998); and 2) IL-10 blocks DNA-binding of NFκB that is already present in the nucleus (Schottelius et al, 1999). Since the inhibition of nuclear NFκB could not be explained by an increase of nuclear levels of its inhibitor IκB (Schottelius et al, 1999), the mechanisms underlying this observation needs to be further investigated.

## 1.7 Function of IL-10

### 1.7.1 Effects of IL-10 on immune cells in vitro

Antigen-presenting cells and lymphocytes are the primary targets of IL-10. Direct effects on these populations explain the major immunological impact of this cytokine, including the regulation of the Th1/Th2 balance (Asadullah et al, 2003). IL-10 promotes the development of a type 2 cytokine pattern, which inhibits the IFN-γ production of T lymphocytes via the suppression of IL-12 synthesis in accessory cells. IL-10 co-stimulates the proliferation and differentiation of B-cells,

which is important in the mounting of adequate defense against intestinal parasites, neutralization of bacterial toxins, and in local mucosal defenses (Romagnani, 1995). Moreover, IL-10 suppresses pro-inflammatory cytokine production and the antigen-presenting capacity of monocytes/macrophages and dendritic cells (De Waal Malefyt et al, 1991; Fiorentino et al, 1991). Therefore, IL-10 acts as a substantial suppressor of the cellular immunity (Spits and De Waal Malefyt, 1992).

### 1.7.2 Effects of IL-10 on B-cells

The effects of IL-10 on the survival, proliferation and differentiation of human B-cells have been extensively studied. IL-10 enhances survival of normal human B-cells, which correlates with the increased expression of the anti-apoptotic protein, Bcl-2 (Levy and Brouet, 1994). IL-10, together with soluble anti-IgM, can upregulate telomerase activity in activated B-cells (Hu et al, 1999). IL-10 is a potent cofactor for proliferation of human B-cell precursors and mature B-cells activated by anti-IgM or CD40 cross-linking (Rousset et al,1992; Saeland et al, 1993). This IL-10 induced proliferation of activated B-cells was further enhanced by both IL-2 and IL-4. The synergistic effect with IL-2, in particular, correlates with IL-10 enhanced expression of high-affinity IL-2 receptor on these B-cells. (Fluckiger et al, 1993).

IL-10 also affects B-cell in differentiation. In long-term culture of B-cells

stimulated by either anti-CD40, activated T-cells or follicular dendritic cells, addition of IL-10 induces differentiation of B-cells into plasma cells (Rousset et al, 1995). IL-10 acts synergistically with CD27/CD70 signals to induce plasma cell differentiation from $CD27^+$ memory B-cells (Agematsu et al, 1998). IL-10 is a very important cytokine for B-cell function.

## 1.8 IL-10 and IL-10 receptor in malignant diseases

### 1.8.1 Melanoma

It has been demonstrated that significant IL-10 mRNA expression is found in melanoma and melanoma metastases but not in healthy skin. This suggests that melanoma cells themselves are contributing at least in part to the IL-10 overexpression (Kruger-Kraskagakes et al, 1994). The overexpression of IL-10 may be of particular pathogenetic importance. IL-10 functions as autocrine growth factor for malignant melanoma and reduces the expression of HLA class I and II on melanoma cell (Yue et al, 1997). IL-10 stimulates the growth of melanoma cell and, in addition, masking the tumor cells from the host immune system.

### 1.8.2 Carcinoma

There are reports on the overexpression of IL-10 in basal cell and squamous cell carcinoma of the skin (Kim et al, 1995). Cytotoxic T-cell lines recognizing these tumors proliferated in the presence of the tumor cells only when IL-10 was neutralized by monoclonal antibodies. On the other hand, the intralesional injection of IFN-α resulted in tumor regression that was associated with the down-regulation of IL-10 mRNA expression. IL-10 seems to be an important mediator in evading the T cell-mediated immune response in these cutaneous malignancies (Kim et al, 1995).

Little is known about IL-10 in other types of epithelial malignancies. It has been demonstrated that -1087 AA genotype is associated with the renal cell carcinoma, however the biological and clinical significances for this observation remain unknown (Havranek et al. 2005).

### 1.8.3 Lymphoma

Tumor cells from B, T, and NK cell lymphoma are able to produce biologically active IL-10 (Kitabayashi et al, 1995; Masood et al, 1995; Sjoberg et al, 1996; Beatty et al, 1997; Boulland et al, 1998; Jones et al, 1999). As early as 1993, elevated IL-10 serum levels were found in patients with active NHL. Elevated IL-10 is detected in serum from about 50% of these NHL patients. IL-10 was detectable with a similar

frequency in all subtypes of NHL and in all clinical stages, as well as in both EBV-seropositive and EBV-seronegative patients (Blay et al, 1993). Moreover, this elevated serum IL-10 levels are associated with poor prognosis of the NHL patients (Blay et al, 1993). These observations suggested that the elevation of serum IL-10 levels in NHL is associated with the presence of an active disease. In the following years these observations were extended to HD and more lymphoma types. Lymphoma patients have significantly higher serum levels of IL-10 than healthy volunteers (Cortes et al, 1995; Cortes and Kurzrock, 1997; Sarris et al, 1999; Bohlen et al, 2000; Vassilakopoulos et al, 2001; Fayad et al, 2001).

In different lymphomas, increase IL-10 production is related to negative prognosis of the patients. (Blay et al, 1993; Stasi et al, 1994; Cortes et al, 1995). Elevated IL-10 serum levels have been described as a negative prognostic factor for responsiveness toward treatment, disease-free duration and the overall survival with extranodal lesions. After radially resection of the primary lesion, the serum levels of IL-10 return to normal in some of these lymphoma patients. Persistently elevated IL-10 serum levels after surgery predicts tumor recurrence (Galizia et al, 2002; Uwatoko et al, 2002). Moreover, a further significant increase in IL-10 serum levels has been observed in non-responders after chemotherapy (Wojciechowska-Lacka et al, 1996; Nemunaitis et al, 2001).

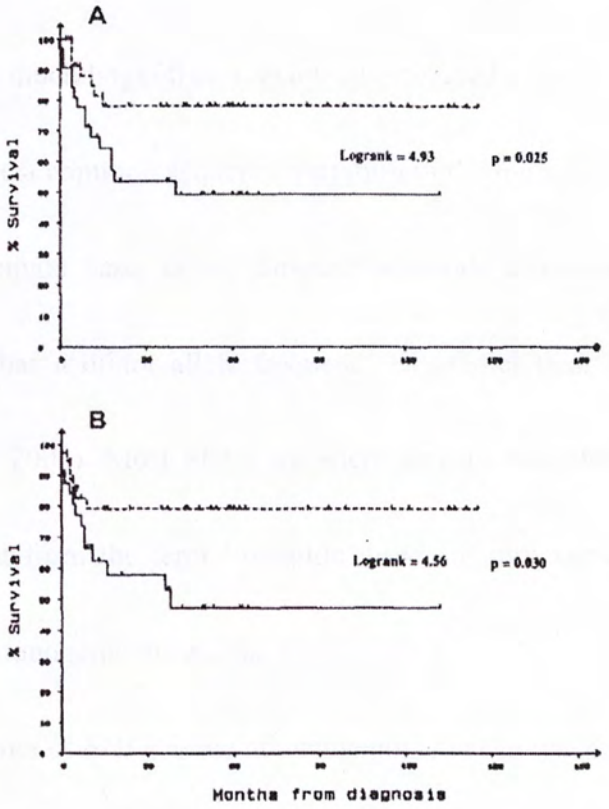



**Fig 1.6** Survival of patients with intermediate or high grade lymphoma. **A,** Overall survival. **B,** Progression free survival.

------- : patients (n=38) without detectable IL-10 at diagnosis,

——— : patients (n=32) with detectable IL-10 at diagnosis. (Blay et al, 1993)

### 1.9 Single Nucleotide Polymorphism (SNP)

One important observation in the human genome project is that the extent of genetic variation is much larger than previously estimated (Lander et al, 2001; Venter et al, 2001). The most common sequence variation in the human genome is the stable substitution of a single base, called Single Nucleotide Polymorphism (SNP). By definition, a SNP has a minor allele frequency of greater than 1% in at least one population (Risch, 2000). Most SNPs are silent and do not alter the function of a gene. It is different from the term 'mutation' used for rare variants, such as those found in classical monogenic disorders.

The total number of SNPs in the human genome is estimated to be more than 10 million (Botstein and Risch, 2003). The number of SNPs with minor allele frequency of over 10% is estimated to be as many as five million (Kruglyak and Nickerson, 2001). SNPs are distributed throughout the human genome, at an estimated overall frequency of at least one in every 1000 base pair (bp) (Carlson et al, 2003). There are, however, marked regional differences of the density of SNP in the human genome. SNP arises because of point mutations that are selectively maintained in populations. The SNP frequencies are determined by: (1) the amount of time elapsed since the variation occurred; (2) evolutionary pressure on biologically significant variants and those linked to the functional variants; (3) random genetic drift; and (4) bottleneck

events (Erichsen and Chanock, 2004).

Single nucleotide polymorphisms in the same chromosomal region are not inherited randomly. They do so as combinations of alleles, which form haplotype blocks. It appears that the genome is organized into distinct blocks of linkage disequilibrium (LD), intercepted by regions in which LD breaks down rapidly (Bonnen et al, 2002; Sabeti et al, 2002). Thus the complexity of analyzing SNPs in a gene or locus can be reduced by the analysis of markers inherited on a haplotype. It is notable that both the frequency of SNPs and the extent of LD may vary significantly between populations. In addition, there are many population-specific private variants (Carlson et al, 2003).

### 1.9.1 SNPs in cancer research

Genetic association studies with SNPs targeting cancer can be divided into two broad categories: investigation of susceptibility or the association with outcomes. The latter seeks to determine prognostic information for survival, complications or response to pharmacological intervention.

#### 1.9.1.1 Susceptibility to cancer and SNPs

The aetiology of a specific cancer is probably associated with a set of genetic variants, many of which could adversely interact with environmental factors. It is notable that some SNPs or haplotypes can be protective. Other SNPs may confer increased susceptibility. For example, certain SNPs in the myeloperoxidase promoter will lead to low risk for developing lung cancer (London et al, 1997). Some SNPs in N-acetyltransferase 1 gene will lead to higher risk to develop bladder cancer (Hein, 2002).

#### 1.9.1.2 Outcome and SNPs

Pharmacogenomics is the study of the inherited basis of inter-individual differences in drug response. It has been estimated that inherited differences account for inter-individual variations in drug responses (Kalow et al, 1998).

Investigations for candidate genes have focused on those genes related to drug metabolism. This includes studies of genes involved in the uptake, activation, degradation and excretion of drugs. The aims are to identify SNPs that are associated with life-threatening adverse reactions. Armed with knowledge of these validated SNPs and haplotype markers that are associated with adverse drug reactions, clinicians could perform screening tests to identify patients at risk. If indicated,

tailored therapy can be designed for patients with specific susceptibility to adverse drug reactions.

### 1.10 SNP in the IL-10 promoter

A number of polymorphisms of the IL-10 gene promoter have been described. There are 3 well-studied single nucleotide polymorphic positions in IL-10 gene promoter that are related to the secretion of IL-10. These SNPs are located at positions -1087 (A/G), -824 (T/C) and -597 (A/G) upstream of the coding region. These three dimorphisms exhibit strong linkage disequilibrium and occur in three putative haplotypes: ACC (A at position -1087; C at -824 and -597), ATA and GCC (Eskdale and Gallagher, 1995).

In vitro stimulation of peripheral blood lymphocytes using concanavalin A revealed that the -1087 GG allele is associated with 1.3 fold increase in IL-10 protein production compared to the low IL-10-producing AA genotype (Eskdale and Gallagher, 1995). The GCC haplotype also exhibits significantly higher transcriptional activity than the ATA haplotype in a luciferase reporter system (Crawley et al, 1999). In lymphoma, genetic variation of the IL10- promoter may also be related to IL-10 production. IL-10 might be produced by both the lymphomas as well as the bystander reactive cells. The IL-10 serum levels reflect the tumor

burden and the host-tumor relationship. (Benjamin et al, 1992; Emilie et al, 1992). In vitro studies revealed that IL-10 production could be related to its gene promoter polymorphisms in lymphoma (Turner et al, 1997). Several studies performed in different ethnic groups indicated that distinct IL-10 alleles or haplotypes are a key factor of IL-10 production in vivo (Eskdale et al, 1997; Lim et al, 1998; Cavet et al, 1999; Helminen et al, 1999). These observations raise the possibility that susceptibility and clinical course of disorders in which immune activation plays an important pathogenic role could be related to the genetic control of IL-10 production.

**1.11 IL-10 promoter SNP in DLBCL**

The role of IL10, SNP in IL-10 promoters and outcomes of DLBCL remains controversial. The IL-10 SNP -1087G allele is associated with DLBCL as compared with the ethnically matched healthy control group in a French series involving 199 patients. The presence of the IL-10 -1087G allele may contribute to the genetic background of DLBCL occurrence (Lech-Maranda et al, 2004). This IL-10 -1087G was previously found to be associated with high IL-10 producing capability (Eskdale et al, 1997; Lim et al, 1998; Hulkkonen et al, 2001). Increased IL-10 production within tumor microenvironment might be of protective value to the tumor cells. IL-10 strongly inhibited the proliferation of T-cell clones via its downregulatory

effects on APC function (Moore et al, 2001). This may facilitate the tumor cell in escaping from the immune system. IL-10(-1087G) allele is noted to be an independent variable, predicting longer freedom from progression in DLBCL with matched IPI. On the other hand, in a Sweden series, involving 168 primary DLBCL and 67 secondary DLBCL, such observation for elevated serum IL-10 cannot be confirmed (Berglund et al, 2005).

# Chapter 2: Aims of Study

**Background:**

SNPs at the IL-10 promoter positions -597, -824 and -1087 are related to the serum level of IL-10. IL-10 is an important immuno-regulatory cytokine of B-cells, and plays an important role in immunosuppression of proinflammatory T-cell. DLBCL is the most common type of non-Hodgkin's Lymphoma (NHL), comprising 30-40% of NHL cases worldwide. Subtyping of DLBCL according to gene expression at the mRNA and protein levels is of prognostic significance. The relationship of SNP of IL-10 promoter, subtypes of DLBCL and prognosis in the local population are not fully understood.

**Objective of the study:**

The aim of this project is to investigate the association of the IL-10 promoter SNPs with NHL and DLBCL. The IL-10 promoter SNPs in specific subtypes of DLBCL is investigated. The prognostic values of in the overall survival of DLBCL.

1. To study the SNP profile in IL-10 promoter at position -597, -824 and -1087 in normal control, NHL patients and DLBCL patients.

2. To sub-classify the archival DLBCL cases by immunohistochemistry.

3. To study the association of DLBCL biomarkers and each IL-10 promoter SNP with overall survival analysis.

# Chapter 3: Materials and Methods

## 3.1 Sample Recruitment

Recruitment of subjects followed the guidelines of local ethical committee and ethical approvals were obtained when required. One hundred and thirty-nine buffy coats from the health blood donors were obtained from the Hong Kong Red Cross for this study in 2003. A total of 144 non-Hodgkin's lymphoma (NHL, from 1987 to 2003) and 108 specimens of diffuse large B-cell lymphoma (DLBCL, from 1995 to 2005) with formalin-fixed, paraffin-embedded archival blocks in the Prince of Wales Hospital, Shatin, Hong Kong, were recruited for this study. The diagnosis of each patient were histologically reviewed and confirmed independently by three histopathologists.

## 3.2 DNA preparation for Single Nucleotide Polymorphism (SNP) analysis

### 3.2.1 Isolation of Peripheral Blood Mononuclear Cell (PBMC) from buffy coat from blood of normal control group

Mononuclear cells, which contain genomic DNA, were isolated by PBMC purification method. Briefly, the buffy coat was diluted with an equal volume of PBS and then overlaid on one-third volume of Ficoll-Paque Plus, density 1.077

(Amersham Biosciences, UK). The buffy coat was centrifuged at 400x g for 30 minutes at room temperature. The mononuclear cells from the interface were collected and washed with 10ml of PBS twice, by centrifuging at 60x g, to remove the platelets. The cell number and the viability were determined by trypan blue exclusion test.

### 3.2.2 Preparation for NHL and DLBCL samples from paraffin-embedded sections for DNA extraction

Ten 20μm-sections of each block was cut and collected into 1.5ml eppendoff microcentrifuge tube. The paraffin sections were then de-waxed by washing with 1.2ml xylene twice, then centrifuged at 13,000 rpm for 5 minutes. After removing the supernatant, the pellet was washed with 1.2ml 100% ethanol twice to remove the remaining xylene, then centrifuged at 13,000 rpm for 5 minutes and the supernatant was removed. The pellet was air-dried at 37°C for 15 minutes.

### 3.2.3 DNA extraction for SNP analysis

DNA was extracted from de-waxed paraffin sections or PBMC isolated from buffy coat of healthy donors using a standard phenol-chloroform method (Sambrook et at, 2001). The specimens were digested with 1mg/ml proteinase K and 2% (w/v)

SDS in STE buffer at 56°C. DNA was then extracted with an equal volume of 24:25:1 phenol:chloroform:isoamy-alcohol (Amresco, Solon, OH). After centrifugation, DNA from the aqueous layer was precipitated with 3 volumes of 100% ethanol and one-third volume of 3M sodium acetate solution, pH 4.2. The precipitation was carried out at -20°C overnight. The precipitated DNA was centrifuged at 13,000 rpm in a micro-centrifuge at room temperature. After discarding the supernatant, the DNA pellet was washed once with 75% ethanol and air-dried. The pellet was resuspended in 50µl TE solution at 56°C for 1 hour. The amount of DNA was quantified using Beckman DU650 spectrophotometer. The concentration (µg/µl) of DNA was calculated by: OD260 x dilution factor x 50/1000.

### 3.3 SNP analysis by Restriction Fragment Length Polymorphism (RFLP)

#### 3.3.1 Amplification of target site by PCR

The genotype of both the patients and normal controls in IL-10 promoter SNP positions -1087, -824 and -597 was analyzed by PCR followed by restriction fragment length polymorphism. The primers were designed with the web-based software "primer3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Table 3.1). PCR reaction was carried out using AmpliTaq Gold reagents (Applied Biosystems, Foster City, CA). In each reaction, 1µl of DNA was added to PCR

mixture containing 1X reaction buffer II (10mM Tris-Cl pH 8.3 and 500mM KCl), 200nM of each of the primer, 2.5mM $MgCl_2$, 1U AmpliTaq Gold and 200µM each of dATP, dCTP, dGTP and dTTP (Amersham Bioscience, UK) in 25µl final reaction volume. The PCR thermocycling was carried out using a GeneAmp9700 PCR system (Applied Biosystems, Foster City, CA). The reaction was heated to 95°C for 10 minutes, followed by cycling at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds for 40 cycles. The PCR product was resolved in 2% agarose gel in TBE. The gel was then stained with ethidium bromide and visualized under UV illumination.

| Primer Name | Direction | Sequence (5'-3') | Amplicon size (bp) |
|---|---|---|---|
| IL-10(597) | Forward | AAT GAA ATC GGG GTA AAG GA | 150 |
| | Reverse | CCT TAG TTT CCC CAA GTA AA | |
| IL-10(824) | Forward | GAA ACC AAA TTC TCA GTT GG | 150 |
| | Reverse | GTC TGC ACT TGC TGA AAG CT | |
| IL-10 (1087) | Forward | CAA GAC AAC ACT ACT AAG GCT TCT T | 100 |
| | Reverse | GTC CCT TAC TTT GCT CTT ACC TAT C | |

**Table 3.1** Primer sequences for Restriction fragment length polymorphism.

### 3.3.2 SNP analysis

The target sequence of -1087, -824 and -597 in IL-10 promoter were analyzed by a web-based software "NEBcutter v2.0" (http://tools.neb.com/NEBcutter2/index.php) to check for the suitable restriction endonucleases for each position. Three restriction endonucleases were used to perform RFLP in the IL-10 promoter SNP analysis, namely, Mnl I (New England Biolabs, Beverly) for position -1087 (A/G), Mae III (Roche Applied Science, Penzberg, Germany) for position -824 (T/C) and Rsa I (Roche Applied Science, Penzberg, Germany) for position -597 (A/C). The recognition sequence of each restriction endonuclease is listed in Table 3.2.

| **Restriction Endonuclease** | **Cutting genotype** | **Recognition sequence** | **Working temperature** |
|---|---|---|---|
| Mnl I | -1087G | 5'…CCTT$(N)_7$^…3'<br>3'…GGAA$(N)_6$^…5' | 37°C |
| Mae III | -824C | 5'…GT^AC…3'<br>3'…CA^TG…5' | 55°C |
| Rsa I | -597A | 5'…^GTNAC…3'<br>3'……CANTG^…5' | 37°C |

**Table 3.2** The recognition sequences and working temperatures of restriction endonucleases for RFLP

The PCR products underwent restriction digestion by specific restriction endonuclease for each IL-10 promoter SNP position. For position -1087, in each

reaction, 10μl of PCR product was added to restriction digestion mixture containing 1X NEBuffer 2 (10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$ and 1mM Dithiothreitol, pH 7.9), 100μg/ml BSA and 1U Mnl I in 20μl final reaction volume. The reaction mixture was then incubated at 37°C water bath overnight. For position -824, in each reaction, 10μl of PCR product was added to restriction digestion mixture containing 1X incubation buffer (20mM Tris-HCl, 275mM NaCl, 6mM $MgCl_2$ and 7mM 2-Mercaptoethanol pH 8.2) and 1U Mae III in 25μl final reaction volume. The reaction mixture was then incubated at 55°C water bath overnight. For position -597, in each reaction, 10μl of PCR product was added to restriction digestion mixture containing 1X SuRE/Cut Buffer L (10mM Tris-HCl, 10mM $MgCl_2$ and 1mM Dithioerythritol pH 7.5) and 1U Rsa I in 20μl final reaction volume. The reaction mixture was then incubated at 37°C water bath overnight. The restriction digested products were resolved in 10% polyacrylamide gel in TBE, stained with ethidium bromide and then visualized under UV illumination. Fig 2.1 shows the electrophoresis profile of different genotypes at position -597, Rsa I will cut at -597 A-allele to form 2 fragments with 50bp and 100bp. For position -824, Mae III will cut in the same electrophoresis profile will be obtained, while for position -1087, the fragments will be cut in 75bp and 25bp by Mnl I. The electrophoresis profile is shown in Fig. 2.2 and 2.3 respectively.

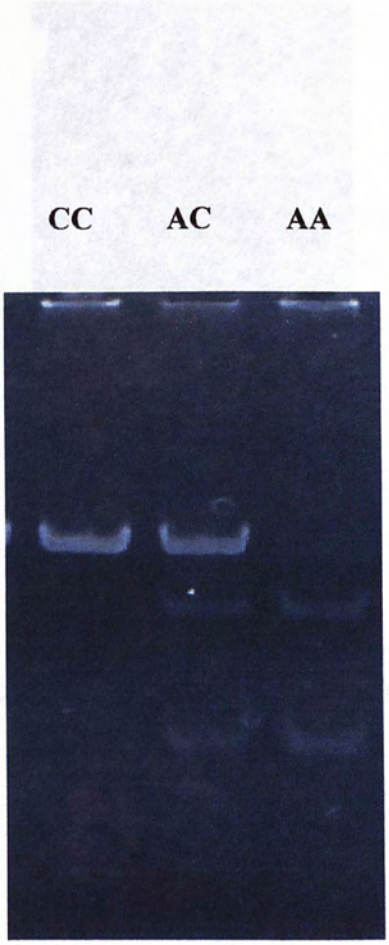



**Fig 2.1** Electrophoresis profile of genotypes at -597


**Fig 2.2** Electrophoresis profile of genotypes at -824


**Fig 2.3** Electrophoresis profile of genotypes at -1087

### 3.4 Determination of haplotypic frequency

The haplotypic frequency of IL-10 promoter was determined by software called "EH". The frequency of all possible haplotypes was input into this software, and the haplotypic frequency was calculated.

### 3.5 Classification of DLBCL by immunohistochemistry

In order to classify different subtypes of DLBCL, immunohistochemistry was performed by staining with 4 different antibodies against CD10, Bcl-6, CD138 and MUM1/IRF4 (Chang et al, 2004). Briefly, 4μm-sections of each sample was cut and mounted on a Lysine-A coated glass slide. The section was deparaffinized in xylene for 3 minutes twice following by rehydration through graded alcohol (100% ethanol, 95% ethanol) and finally distilled water, 3 minutes for each step.

Antigen retrieval was performed using high temperature antigen unmasking method under microwave. Slides after rehydration were put into 1mM Citrate Buffer. Slides were then heated in a domestic microwave (Panasonic, 800W) oven twice, using Power 10 (800W) for 3 minutes and then Power 3 (250W) for 10 minutes. After microwave treatment, slides were cooled down for 30 minutes under room temperature to prevent the detachment of section from glass slide.

After antigen retrieval, the slides were pretreated in 0.3% $H_2O_2$ for 10 minutes to block the endogenous peroxidase by substrate-inhibition. Slides were then washed for three times with TBS for 5 minutes. Slides were then blocked with 5% v/v normal rabbit serum for 10 minutes. Excess normal rabbit serum was removed after blocking, and replaced by primary mouse anti-human antibody, anti-CD10 (Zymed Laboratories, South San Francisco, CA), anti-Bcl-6 (DakoCytomation, Denmark), anti-CD138 (DakoCytomation, Denmark) and anti-MUM1/IRF4(DakoCytomation, Denmark), diluted in TBS with 0.3% BSA with dilution of 1:100. The slides were incubated at room temperature overnight.

After incubation, slides were washed three times with TBS for 5 minutes. Biotinylated rabbit anti-mouse secondary antibody (DakoCytomation, Denmark), diluted 1:200 in TBS with 0.3% BSA, was added to the slides and then incubated for 30 minutes at room temperature. Slides were then washed with TBS three times for 5 minutes.

Streptavidin-HRP (Zymed Laboratories, South San Francisco, CA), diluted in 1:400 with TBS and 0.3% BSA, were added to slides and incubated for 45 minutes at room temperature. The slides were then washed with TBS three times for 5 minutes. 3,3'-Diaminobenzidine (DAB) development was performed by adding DAB solution which contain 0.2ml 2.5% DAB dissolved in 10ml TBS with 0.3% $H_2O_2$. DAB

development lasted for 10 minutes, and brown color was observed for positive staining. After DAB development, the slides were counterstained with Haematoxylin. The slides were dehydrated through graded alcohol (95% ethanol and then 100% ethanol), and finally in xylene for 3 times, 3 minutes for each step. The slides were mounted and ready for scoring.

Immunohistochemical stained slides were independently scored by 3 investigators Dr. Anthony Lo, Dr. Amy Chan and me. Immunohistochemical stainings for each of the antibodies were positive if more than 20% of the lymphoma cells showed the expected patterns of staining. The staining intensity was not included in the diagnosis of positivity. Conflicts of interpretation between investigators were resolved with resolution by the aid of multi-head microscope. Subtyping of DLBCL follows Table 1.1 in the Introduction.

### 3.5.1 Staining pattern of CD10

A membranous staining pattern is observed and this staining pattern is usually homogenous throughout the tumour, Fig 3.1.

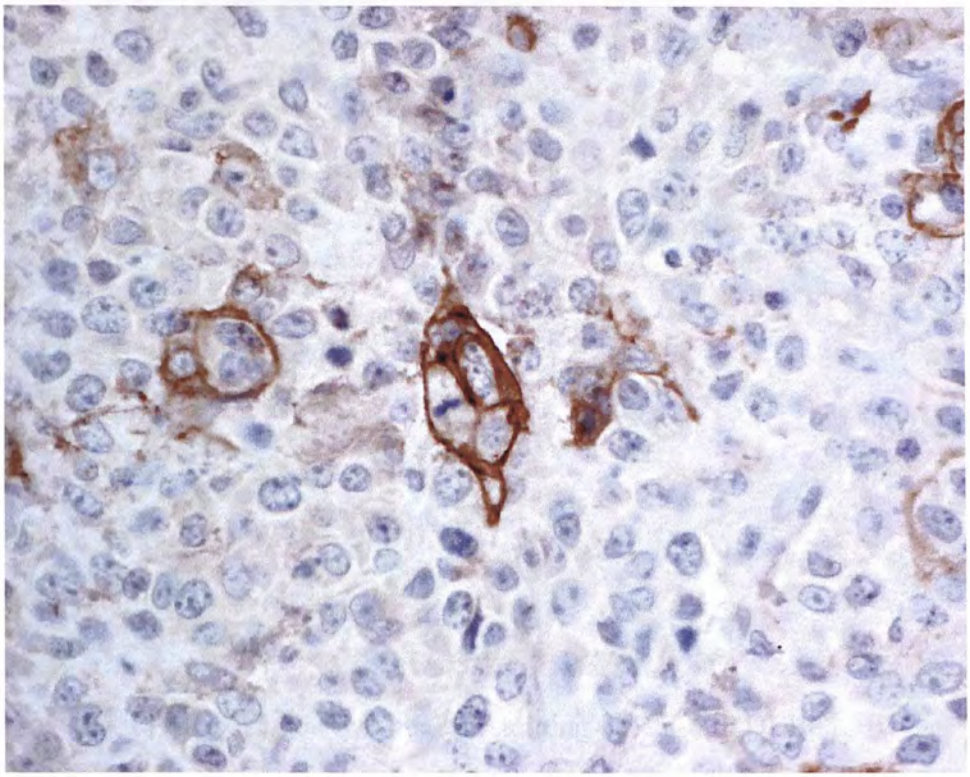


**Figure 3.1** Positive membranous staining of CD10 in DLBCL

### 3.5.2 Staining pattern of Bcl-6

A nuclear staining is obtained by immunohistochemistry, Fig. 3.2.

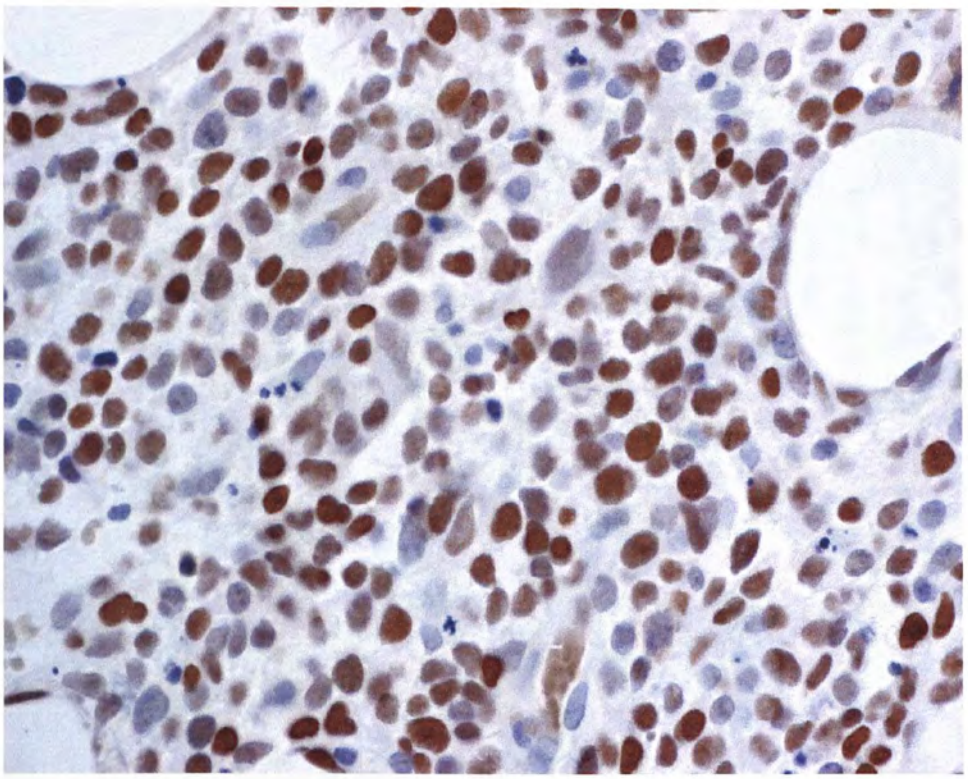


**Figure 3.2** Positive nuclear staining of Bcl-6 in DLBCL

### 3.5.3 Staining pattern of CD138

Expression of CD138 shows a cytoplasmic/membranous staining pattern by immunhistochemistry, Fig. 3.3.

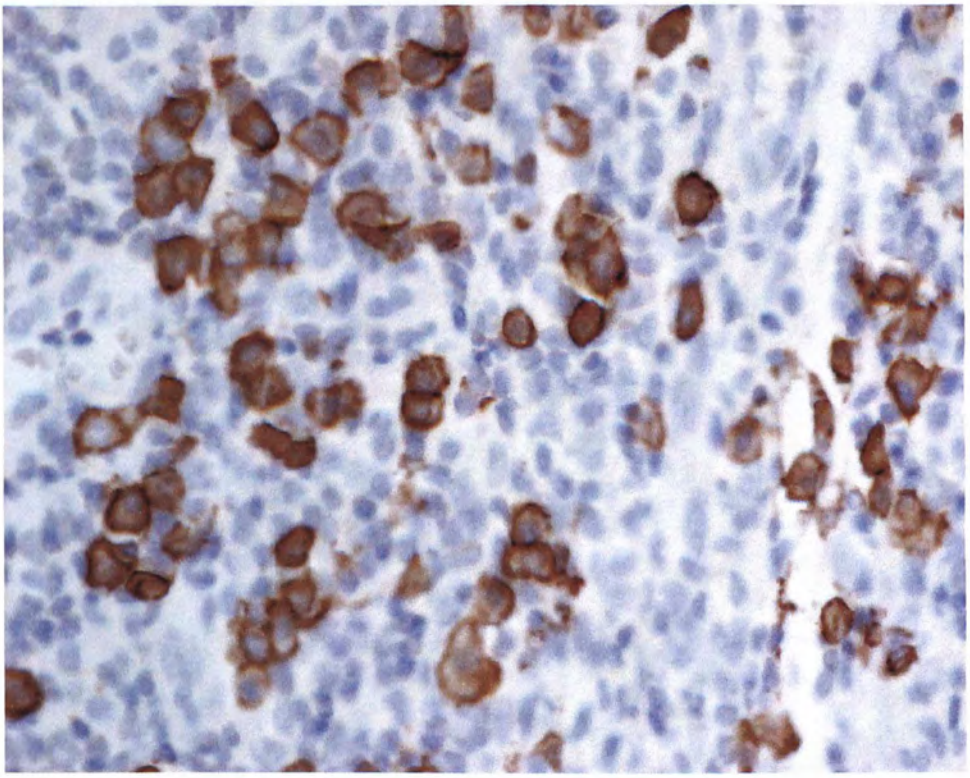


**Figure 3.3** Positive cytoplasmic/membranous staining of CD138 in DLBCL

### 3.5.4 Staining pattern of MUM1/IRF4

Expression of MUM1/IRF4 shows a cytoplasmic/nuclear staining pattern by immunhistochemistry, Fig. 3.4.

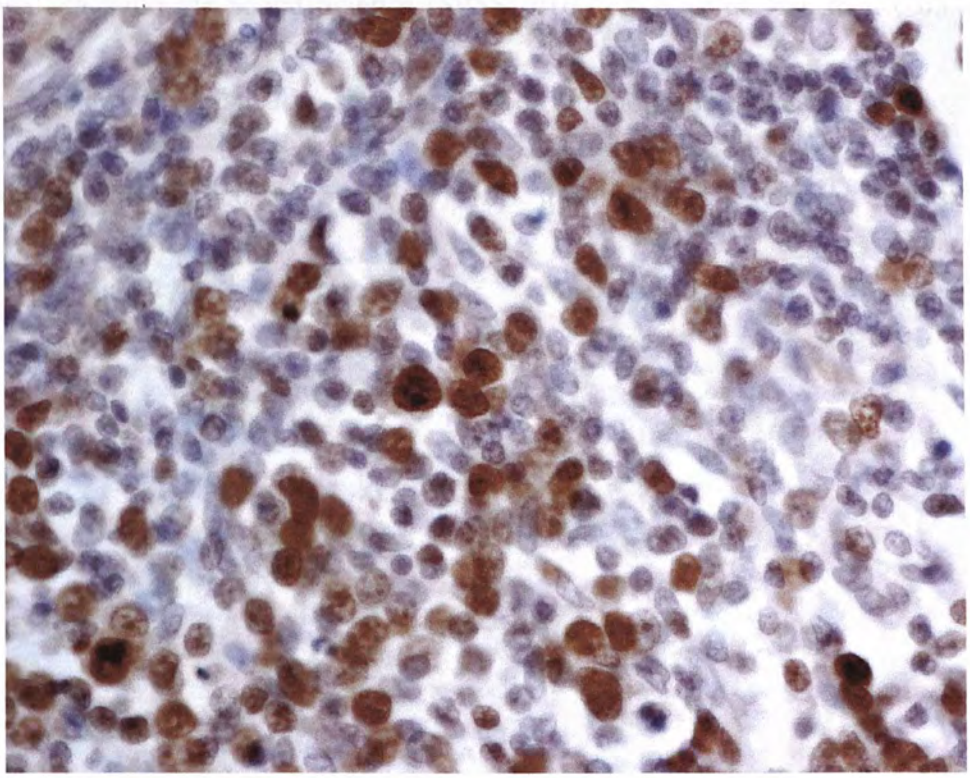


**Figure 3.4** Positive cytoplasmic/nuclear staining of MUM1/IRF4 in DLBCL.

### 3.6 Statistical Analysis

Chi-square test was applied for the statistical analysis in IL-10 promoter SNP study for sample size larger than 5. For those group which the sample size less than 5, Fisher exact test was used to calculate the $p$ value. The data was processed using Statistical Package for Social Science (SPSS) v10.1. A $p$ value less than 0.05 was considered to be statistically significant. Cox Proportional Hazards Regression Analysis was also processed by SPSS v10.1 for the overall survival analysis of DLBCL patients.

# Chapter 4: Results

## 4.1 SNPs of IL-10 promoter in normal controls

### 4.1.1 Allelic Frequencies and genotype distributions

The buffy coats were collected from the Hong Kong Red Cross in the year 2003. All subjects were anonymous. No epidemiological information was available, except that all the specimens were from the local Chinese donors in Hong Kong. The allelic frequencies and genotypes distribution at positions -1087, -824 and -597 of the IL-10 promoter in 139 normal healthy controls are shown in Table 4.1. The most common alleles at the IL-10 promoter were -1087 A (97.48%), -824 T (70.14%) and -597 A (70.14%). The most common genotypes at IL-10 promoter positions -1087, -824 and -597 were AA (94.96%), TT (51.08%) and AA (51.08%), respectively. Interestingly, the genotypes at positions -824 and -597 are closely linked. The -824 T-allele was followed by -597 A-allele; and -824 C-allele was followed by -597 C-allele. No linkage was observed in position -1087 with the other two positions.

### 4.1.2 Haplotypic Frequencies of normal controls

The haplotypic frequencies of IL-10 promoter in 139 normal controls are shown in Table 4.2. Four haplotypes were observed: ATA (69.78%), ACC (27.70%), GCC

(2.16%) and GTA (0.36%). The halpotypes ATC, ACA, GTC and GCA were not detected in this control group.

## 4.2 SNP of the IL-10 promoter in non-Hodgkin's lymphomas

### 4.2.1 Allelic frequencies and genotype distributions

The allelic frequencies and genotype distributions at position -1087, -824 and -597 of IL-10 promoter in normal healthy control group and 144 NHL patients are shown in Table 4.1. NHL patients had the same common alleles and similar allelic frequencies. The specific genotypes encountered and the distribution in NHL was the same as those of the normal controls. As shown by the Chi-square test, both allelic frequencies and genotype distributions of NHL patients were not statistically differenct from those of the normal control.

| | Control (n=139) | NHL (n=144) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 3.47% | 0.339 |
| IL-10 $_{-1087A}$ | 97.48% | 96.53% | 0.339 |
| IL-10 $_{-824T}$ | 70.14% | 73.38% | 0.857 |
| IL-10 $_{-824C}$ | 29.86% | 26.62% | 0.857 |
| IL-10 $_{-597A}$ | 70.14% | 73.38% | 0.857 |
| IL-10 $_{-597C}$ | 29.86% | 26.62% | 0.857 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 0 (0.00%) | N/A |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 10 (6.94%) | 0.499 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 134 (93.06%) | 0.499 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 69 (47.92%) | 0.595 |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 66 (45.83%) | 0.189 |
| IL-10 $_{-824CC}$ | 15 (10.79) | 9 (6.25%) | 0.170 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 69 (47.92%) | 0.595 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 66 (45.83%) | 0.189 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 9 (6.25%) | 0.170 |

**Table 4.1** Allelic frequencies and genotype distributions of IL-10.$_{-1087}$, IL-10.$_{-824}$ and IL-10.$_{-597}$ polymorphisms in 139 normal healthy controls and 144 NHL patients.

[a] Chi-square test comparing the normal controls and NHL. A p-value of <0.05 is considered significant.

### 4.2.2 Haplotypic frequencies

The haplotypic frequencies of IL-10 promoter in 139 normal controls and 144 NHL patients are shown in Table 4.2. The four haplotypes found in the normal subjects were also observed in the NHL patients. No unique halpotype was detected in NHL. The frequency of each halpotype was similar in both NHL and control. As shown by Chi-square test, there were no significant differences between normal control and NHL patients.

| | Control (n=139) | NHL (n=144) | *p*-value[a] |
|---|---|---|---|
| **Haplotypic Frequencies** | | | |
| ATA | 194 (69.78%) | 203 (70.49%) | 0.566 |
| ATC | 0 | 0 | |
| ACA | 0 | 0 | |
| ACC | 77 (27.70%) | 75 (26.04%) | 0.657 |
| GTA | 1 (0.36%) | 1 (0.35%) | 1.000 |
| GTC | 0 | 0 | |
| GCA | 0 | 0 | |
| GCC | 6 (2.16%) | 9 (3.13%) | 0.474 |

**Table 4.2** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 144 NHL patients.

[a] Chi-square test comparing the normal controls and NHL. A p-value of <0.05 is considered significant.

## 4.4 SNPs of the IL-10 promoter in DLBCL

### 4.4.1 Allelic frequencies and genotype distributions

108 DLBCL cases were collected retrospectively from the formalin-fixed, paraffin-embedded archival sections. Among these samples, there are 53 males and 55 females. The mean age was 61.51 years.

The allelic frequencies and genotype distributions at position -1087, -824 and -597 of the IL-10 promoter in normal healthy control group and 108 DLBCL patients are shown in Table 4.3. There were no significant difference between the allelic frequencies in DLBCL patients and normal control in all the three SNP positions. In DLBCL, the most common genotypes at positions -1087 and -597 were AA (97.22%) and AA (48.15%). However, no significant differences were found in the genotype distributions between normal control and DLBCL in these two positions. On the other hand, the most common genotype of DLBCL at position -824 was TC (57.41%), which was different from the normal control. Moreover, significant differences were observed in the genotypes at these positions between DLBCL and normal by Chi-square test: IL-$10_{-824TT}$ ($p = 0.019$) and IL-$10_{-824TC}$ ($p = 0.003$). Furthermore, unlike the case of normal controls, the genotypes of DLBCL at position -824 were not always linked with those at position -597.

| | Control (n=139) | DLBCL (n=108) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 1.85% | 0.619 |
| IL-10 $_{-1087A}$ | 97.48% | 98.15% | 0.619 |
| IL-10 $_{-824T}$ | 70.14% | 64.81% | 0.209 |
| IL-10 $_{-824C}$ | 29.86% | 35.19% | 0.209 |
| IL-10 $_{-597A}$ | 70.14% | 69.91% | 0.955 |
| IL-10 $_{-597C}$ | 29.86% | 30.09% | 0.955 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 1 (0.93%) | 0.256 |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 2 (1.85%) | 0.185 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 105 (97.22%) | 0.372 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 39 (36.11%) | 0.019* |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 62 (57.41%) | 0.003* |
| IL-10 $_{-824CC}$ | 15 (10.79) | 7 (6.48%) | 0.238 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 52 (48.15%) | 0.648 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 47 (43.52%) | 0.392 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 9 (8.33%) | 0.518 |

**Table 4.3** Allelic frequencies and genotype distributions of IL-10.$_{-1087}$, IL-10.$_{-824}$ and IL-10.$_{-597}$ polymorphisms in 139 normal healthy controls and 108 DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.4.2 Haplotypic frequencies

The haplotypic frequencies of the IL-10 promoter in 139 normal controls and 108 DLBCL patients are shown in Table 4.4. Besides the haplotypes ATA, ACC, GTC and GCC that were also observed in normal controls, haplotypes ATC, ACA and GCA were also detected in patients with DLBCL. As shown in Chi-square test, significant differences was obtained in haplotypes ACA (p = 0.000).

| | Control (n=139) | DLBCL (n=108) | *p*-value[a] |
|---|---|---|---|
| **Haplotypic Frequencies** | | | |
| ATA | 194 (69.78%) | 136 (62.96%) | 0.089 |
| ATC | 0 | 2 (0.93%) | 0.191 |
| ACA | 0 | 12 (5.56%) | 0.000 * |
| ACC | 77 (27.70%) | 62 (28.70%) | 0.894 |
| GTA | 1 (0.36%) | 1 (0.46%) | 1.000 |
| GTC | 0 | 0 | N/A |
| GCA | 0 | 1 (0.46%) | 1 |
| GCC | 6 (2.16%) | 4 (15.38%) | 1.000 |

**Table 4.4** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 108 DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.5 SNP of the IL-10 promoter in different subtypes of DLBCL

#### 4.5.1 Classification of DLBCL by immunohistochemistry

DLBCL were subclassified into different subtypes according to the immunohistochemical stainings of antibodies against CD10, Bcl-6, CD138 and MUM1 (Table 1.1 in Introduction). There were 23 cases positive for CD10; 41 cases positive for Bcl-6; 25 cases positive for CD138 and 94 cases positive for MUM1 (Table 4.5). The frequency of each subtypes of DLBCL is shown in Table 4.6. The most common subtype of DLBCL was activated non-germinal center DLBCL which comprised 60 cases (55.56%). Germinal center DLBCL was rare in the local series.

| Antigens | Positive (%) | Negative (%) |
|---|---|---|
| CD10 | 23 (21.30%) | 85 (78.70%) |
| Bcl-6 | 41 (37.96%) | 67 (62.04%) |
| CD138 | 25 (23.15%) | 83 (76.85%) |
| MUM1 | 94 (87.04%) | 14 (12.96%) |

**Table 4.5** Immunohistochemical results with antibodies against CD10, Bcl-6, CD138 and MUM1of 108 DLBCL patients.

| DLBCL Subtypes | Frequency | Percentage |
|---|---|---|
| Germinal Center | 9 | 8.33% |
| Activated Germinal Center | 28 | 25.93% |
| Activated non-Germinal Center | 60 | 55.56% |
| Unclassified | 11 | 10.19% |
| Total | 108 | 100% |

**Table 4.6** Frequencies of DLBCL subtypes using immunohistochemical classification by 4 antibodies against CD10, Bcl-6, CD138 and MUM1.

### 4.5.2 SNP of the IL-10 promoter in Germinal Center DLBCL (GC-DLBCL)

#### 4.5.2.1 Allelic frequencies and genotype distributions

There were 9 DLBCL patients classified as GC-DLBCL. The allelic frequencies and genotype distributions at positions -1087, -824 and -597 of the IL-10 promoter in normal control group and 9 GC-DLBCL patients are shown in Table 4.7. As shown by Chi-square test, there were no significant differences in allelic frequencies between GC-DLBCL patients and normal control. However, in genotype distributions, significant differences were found in IL-10 $_{-824TT}$ ($p = 0.034$) and IL-10 $_{-824TC}$ ($p = 0.004$).

| | Control (n=139) | GC-DLBCL (n=9) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 0.00% | 0.496 |
| IL-10 $_{-1087A}$ | 97.48% | 100.00% | 0.496 |
| IL-10 $_{-824T}$ | 70.14% | 55.56% | 0.194 |
| IL-10 $_{-824C}$ | 29.86% | 44.44% | 0.194 |
| IL-10 $_{-597A}$ | 70.14% | 72.22% | 0.852 |
| IL-10 $_{-597C}$ | 29.86% | 27.78% | 0.852 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 0 (0.00%) | N/A |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 0 (0.00%) | 0.490 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 9 (100.00%) | 0.490 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 8 (88.89%) | 0.034* |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 1(11.11%) | 0.004 * |
| IL-10 $_{-824CC}$ | 15 (10.79) | 0 (0.00%) | 0.599 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 4 (44.44%) | 0.700 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 5 (55.56%) | 0.299 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 0 (0.00%) | 0.599 |

**Table 4.7** Allelic frequencies and genotype distributions of IL-10.$_{-1087}$, IL-10.$_{-824}$ and IL-10.$_{-597}$ polymorphisms in 139 normal healthy controls and 9 GC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

#### 4.5.1.2 Haplotypic frequencies

The haplotypic frequencies of the IL-10 promoter in 139 normal controls and 9 GC-DLBCL patients are shown in Table 4.8. As shown in the table by Chi-square test, significant difference was found for haplotype ACA ($p < 0.001$).

| Haplotypic Frequencies | Control (n=139) | GC-DLBCL (n=9) | *p*-value[a] |
|---|---|---|---|
| ATA | 194 (69.78%) | 10 (55.56%) | 0.206 |
| ATC | 0 | 0 | N/A |
| ACA | 0 | 3 (16.67%) | 0.000* |
| ACC | 77 (27.70%) | 5(27.77%) | 0.994 |
| GTA | 1 (0.36%) | 0 | 0.798 |
| GTC | 0 | 0 | N/A |
| GCA | 0 | 0 | N/A |
| GCC | 6 (2.16%) | 0 | 0.525 |

**Table 4.8** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 9 GC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.5.2 SNP of the IL-10 promoter in Activated Germinal Center DLBCL (AGC-DLBCL)

#### 4.5.2.1 Allelic frequencies and genotype distributions

There were 28 DLBCL patients classified as AGC-DLBCL. The allelic frequencies and genotype distributions at positions -1087, -824 and -597 of the IL-10 promoter in normal control group and 28 AGC-DLBCL patients are shown in Table 4.9. As shown in the table by Chi-square test, there were no significant differences between normal and AGC-DLBCL patients in both allelic frequencies and genotype distributions.

| | Control (n=139) | AGC-DLBCL (n=28) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 3.57% | 0.657 |
| IL-10 $_{-1087A}$ | 97.48% | 96.43% | 0.657 |
| IL-10 $_{-824T}$ | 70.14% | 62.50% | 0.260 |
| IL-10 $_{-824C}$ | 29.86% | 37.50% | 0.260 |
| IL-10 $_{-597A}$ | 70.14% | 67.86% | 0.734 |
| IL-10 $_{-597C}$ | 29.86% | 32.14% | 0.734 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 0 (0.00%) | N/A |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 2 (7.14%) | 0.652 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 26 (92.86%) | 0.652 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 10 (35.72%) | 0.138 |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 15(53.57%) | 0.129 |
| IL-10 $_{-824CC}$ | 15 (10.79) | 3 (10.71%) | 1.000 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 13 (46.43%) | 0.653 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 12 (42.86%) | 0.640 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 3 (10.71%) | 1.000 |

**Table 4.9** Allelic frequencies and genotype distributions of IL-10.$_{-1087}$, IL-10.$_{-824}$ and IL-10.$_{-597}$ polymorphisms in 139 normal healthy controls and 28 AGC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

#### 4.5.2.2 Haplotypic frequencies

The haplotypic frequencies of IL-10 promoter in 139 normal controls and 28 AGC-DLBCL patients are shown in Table 4.10. As shown by Chi-square test, significant difference was found in haplotype ACA (p = 0.028).

| | Control (n=139) | AGC-DLBCL (n=28) | *p*-value[a] |
|---|---|---|---|
| **Haplotypic Frequencies** | | | |
| ATA | 194 (69.78%) | 34 (60.71%) | 0.183 |
| ATC | 0 | 0 | N/A |
| ACA | 0 | 2 (3.57%) | 0.028* |
| ACC | 77 (27.70%) | 18 (32.14%) | 0.501 |
| GTA | 1 (0.36%) | 1 (1.79%) | 0.308 |
| GTC | 0 | 0 | N/A |
| GCA | 0 | 1 (1.79%) | 0.168 |
| GCC | 6 (2.16%) | 0 | 0.525 |

**Table 4.10** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 28 AGC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.5.3 SNP of the IL-10 promoter in Activated non-Germinal Center DLBCL (ANGC-DLBCL)

#### 4.5.3.1 Allelic frequencies and genotype distributions

There were 60 DLBCL patients classified as ANGC-DLBCL. The allelic frequencies and genotype distributions at positions -1087, -824 and -597 of IL-10 promoter in normal control group and 60 ANGC-DLBCL patients are shown in Table 4.11. As shown in the table by Chi-square test, there were no significant differences between ANGC-DLBCL patients and normal control in allelic frequencies. However, significant difference was found in the genotype IL-10 $_{-824TC}$ ($p = 0.027$).

| | Control (n=139) | ANGC-DLBCL (n=60) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 1.67% | 0.729 |
| IL-10 $_{-1087A}$ | 97.48% | 98.33% | 0.729 |
| IL-10 $_{-824T}$ | 70.14% | 67.50% | 0.600 |
| IL-10 $_{-824C}$ | 29.86% | 32.50% | 0.600 |
| IL-10 $_{-597A}$ | 70.14% | 70.00% | 0.977 |
| IL-10 $_{-597C}$ | 29.86% | 30.00% | 0.977 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 1 (1.67%) | 0.302 |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 0 (0.00%) | 0.105 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 59 (98.33%) | 0.439 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 24 (40.00%) | 0.151 |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 33 (55.00%) | 0.027* |
| IL-10 $_{-824CC}$ | 15 (10.79) | 3(5.00%) | 0.282 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 29 (48.33%) | 0.722 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 26 (43.33%) | 0.491 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 5 (8.34%) | 0.597 |

**Table 4.11** Allelic frequencies and genotype distributions of IL-10.$_{1087}$, IL-10.$_{824}$ and IL-10.$_{597}$ polymorphisms in 139 normal healthy controls and 60 ANGC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

#### 4.5.3.2 Haplotypic frequencies

The haplotypic frequencies of IL-10 promoter in 139 normal controls and 60 ANGC-DLBCL patients are shown in Table 4.12. As shown in the table by Chi-square test, significant differences was been found in haplotype ACA (p = 0.002).

| Haplotypic Frequencies | Control (n=139) | ANGC-DLBCL (n=60) | *p*-value[a] |
|---|---|---|---|
| ATA | 194 (69.78%) | 79(65.83%) | 0.436 |
| ATC | 0 | 2 (1.67%) | 0.090 |
| ACA | 0 | 5 (4.16%) | 0.002* |
| ACC | 77 (27.70%) | 32 (26.67%) | 0.832 |
| GTA | 1 (0.36%) | 0 | 1.000 |
| GTC | 0 | 0 | N/A |
| GCA | 0 | 0 | N/A |
| GCC | 6 (2.16%) | 2 (1.67%) | 1.000 |

**Table 4.12** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 60 ANGC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.5.4 SNP of the IL-10 promoter in Unclassified DLBCL (UC-DLBCL)

#### 4.5.4.1 Allelic frequencies and genotype distributions

There were 11 DLBCL patients that could not be classified as they did not express any of the 4 markers used in this study. The allelic frequencies and genotype distributions at positions -1087, -824 and -597 of IL-10 promoter in normal control group and 11 UC-DLBCL patients are shown in Table 4.13. As shown in the table by Chi-square test, there were no significant differences between the UC-DLBCL patients and the normal control in both allelic frequencies and genotype distributions.

| | Control (n=139) | UC-DLBCL (n=11) | *p*-value[a] |
|---|---|---|---|
| **Allelic Frequencies** | | | |
| IL-10 $_{-1087G}$ | 2.52% | 0.00% | 1.000 |
| IL-10 $_{-1087A}$ | 97.48% | 100.00% | 1.000 |
| IL-10 $_{-824T}$ | 70.14% | 63.64% | 0.523 |
| IL-10 $_{-824C}$ | 29.86% | 36.36% | 0.523 |
| IL-10 $_{-597A}$ | 70.14% | 72.73% | 0.798 |
| IL-10 $_{-597C}$ | 29.86% | 27.27% | 0.798 |
| **Genotype Distributions (%)** | | | |
| IL-10 $_{-1087GG}$ | 0 (0.00%) | 0 (0.00%) | N/A |
| IL-10 $_{-1087GA}$ | 7 (5.04%) | 0 (0.00%) | 1.000 |
| IL-10 $_{-1087AA}$ | 132 (94.96%) | 11 (100.00%) | 1.000 |
| IL-10 $_{-824TT}$ | 71 (51.08%) | 4 (36.36%) | 0.347 |
| IL-10 $_{-824TC}$ | 53 (38.13%) | 6 (54.55%) | 0.283 |
| IL-10 $_{-824CC}$ | 15 (10.79) | 1(9.09%) | 1.000 |
| IL-10 $_{-597AA}$ | 71 (51.08%) | 6 (54.55%) | 0.825 |
| IL-10 $_{-597AC}$ | 53 (38.13%) | 4 (36.36%) | 1.000 |
| IL-10 $_{-597CC}$ | 15 (10.79) | 1 (9.09%) | 1.000 |

**Table 4.13** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 11 UC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

#### 4.5.4.2 Haplotypic frequencies

The haplotypic frequencies of IL-10 promoter in 139 normal controls and 11 UC-DLBCL patients are shown in Table 4.14. As shown in the table by Chi-square test, significant difference was found in haplotype ACA (p = 0.005).

| Haplotypic Frequencies | Control (n=139) | UC-DLBCL (n=11) | *p*-value[a] |
|---|---|---|---|
| ATA | 194 (69.78%) | 14(63.64%) | 0.547 |
| ATC | 0 | 0 | N/A |
| ACA | 0 | 2 (9.09%) | 0.005* |
| ACC | 77 (27.70%) | 6 (27.27%) | 0.966 |
| GTA | 1 (0.36%) | 0 | 1.000 |
| GTC | 0 | 0 | N/A |
| GCA | 0 | 0 | N/A |
| GCC | 6 (2.16%) | 0 | 1.000 |

**Table 4.14** Haplotypic frequencies of IL-10 promoter in 139 normal controls and 11 UC-DLBCL patients.

[a] Chi-square test comparing the normal controls and DLBCL. A p-value of <0.05 is considered significant (*).

### 4.6 Summary of SNP of the IL-10 promoter in DLBCL subtypes

We observed that in AGC-, ANGC- and UC-DLBCL, the most common genotype in IL-10 $_{-824}$ was heterozygous TC. This was different from the normal control in which the common genotype was the homozygous TT. However, in GC-DLBCL the most common genotype for IL-10 -824 was also homozygous TT as the normal control.

We observed that there were rare haplotypes, ATC, ACA and GCA, which only appeared in DLBCLs (Table 4.15). These haplotypes were not found in other ethnic groups, but only in the Chinese population (Hu et al, 2003). In the present series, significant difference was only obtained in haplotype ACA among all DLBCL subtypes.

| | GC-DLBCL | AGC-DLBCL | ANGC-DLBCL | UC-DLBCL | All DLBCL |
|---|---|---|---|---|---|
| **ATC** | 0 | 0 | 2 (100.00%) | 0 | 2 |
| **ACA** | 3 (25.00%) | 2 (16.67%) | 5 (41.67%) | 2 (16.67%) | 12 |
| **GCA** | 0 | 1 (100.00%) | 0 | 0 | 1 |

**Table 4.15** Summary of frequencies of the rare halpotypes found only in DLBCL.

## 4.7 Overall survival analysis

### 4.7.1 Clinical data of DLBCL

Among the 108 DLBCL patients, the clinical data of 58 *de novo* DLBCL patients were available and were retrieved retrospectively. The clinical data obtained included the general demographic data, the survival status, date of diagnosis and date of death (Table 4.16). Among these 58 cases, there were 28 males and 30 females (M:F 1:1.07). The mean age was 60.29 (range: 16-92). Among these primary DLBCLs, 39 (37.20%) cases were located at extra-nodal site and 19 (32.80%) cases were nodal diseases. There were twenty-five of the patients have already died and their mean survival period is 12.72 months (range: 0-124 months). Among these cases, there were 8 (13.80%) positive for CD10; 23 (39.70%) positive for Bcl-6; 12 (20.70%) positive for CD138 and 54 (93.10%) positive MUM1. From immunohistochemical classification, 2 (3.40%) cases were GC-DLBCL, 22 (37.90%) cases were AGC-DLBCL, 33 (56.90%) cases were ANGC-DLBCL and 1 (1.70%) cases were unclassified (Table 4.16).

| DLBCL Subtypes | Frequency | Percentage |
|---|---|---|
| Germinal Center | 2 | 3.45% |
| Activated Germinal Center | 22 | 37.93% |
| Activated non-Germinal Center | 33 | 56.90% |
| Unclassified | 1 | 1.72% |
| Total | 58 | 100% |

**Table 4.16** Frequency of DLBCL subtypes in overall survival analysis

### 4.7.2 Cox Proportional Hazards Regression Analysis in DLBCL

Univariate Cox Proportional Hazards Regression Analysis was done to estimate the impact of expression of each marker and expression patterns of these markers in the primary DLBCL patients (Table 4.17). There were no statistically significant impacts on overall survival observed in expression patterns of CD10, Bcl-6, CD138 and MUM1 as well as the SNPs of IL-10 promoter.

Further statistical modelings were performed to investigate whether there was impact on overall survival of each parameter together with one of the immunohistochemical marker. In this analysis, the expressions of CD10, Bcl-6, CD138 and MUM1 were investigated individually in the impact of overall survival. The results were shown in Table 4.19-22. There were no significant impacts in each parameter, including SNPs of IL-10 promoters and disease sites together with CD10, Bcl-6, CD138 and MUM1 on the overall survival of DLBCL subtypes.

**Table 4.17** Clinical and Immunohistochemical data of DLBCL patients

| Sample No. | Gender | Age | Location[a] | CD10 | Bcl-6 | CD138 | MUM1 | DLBCL Subtype[b] | Survival | Duration[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Male | 68 | E | - | + | - | - | GC | dead | 34 |
| 2 | Male | 90 | E | - | + | - | - | GC | dead | 3 |
| 3 | Female | 68 | E | + | + | + | + | AGC | alive | 56 |
| 4 | Male | 49 | E | + | + | + | - | AGC | alive | 37 |
| 5 | Male | 77 | E | + | + | - | + | AGC | alive | 38 |
| 6 | Female | 61 | E | - | + | - | + | AGC | alive | 38 |
| 7 | Male | 65 | E | + | + | - | + | AGC | alive | 37 |
| 8 | Male | 69 | E | + | + | - | + | AGC | alive | 29 |
| 9 | Male | 75 | N | - | + | - | + | AGC | alive | 28 |
| 10 | Male | 42 | E | + | + | - | + | AGC | alive | 28 |
| 11 | Female | 55 | E | + | - | - | + | AGC | alive | 26 |
| 12 | Female | 37 | N | - | + | - | + | AGC | alive | 21 |
| 13 | Female | 63 | E | - | + | - | + | AGC | alive | 19 |
| 14 | Female | 72 | N | - | + | - | + | AGC | alive | 14 |
| 15 | Female | 70 | E | - | + | - | + | AGC | alive | 8 |
| 16 | Female | 16 | E | - | + | - | + | AGC | alive | 123 |
| 17 | Female | 92 | E | - | + | - | + | AGC | dead | 3 |
| 18 | Male | 56 | N | - | + | - | + | AGC | dead | 5 |
| 19 | Female | 87 | E | - | + | + | + | AGC | dead | 1 |
| 20 | Female | 88 | E | - | + | - | + | AGC | dead | 11 |
| 21 | Female | 81 | E | + | + | - | + | AGC | dead | 9 |

| Sample No. | Gender | Age | Location | CD10 | Bcl-6 | CD138 | MUM1 | DLBCL Subtype | Survival | Duration |
|---|---|---|---|---|---|---|---|---|---|---|
| 22 | Female | 77 | E | - | + | + | + | AGC | dead | 19 |
| 23 | Female | 50 | E | - | + | - | + | AGC | dead | 9 |
| 24 | Male | 55 | N | - | + | - | + | AGC | dead | 11 |
| 25 | Female | 58 | N | - | - | + | + | ANGC | alive | 58 |
| 26 | Female | 38 | N | - | - | + | + | ANGC | alive | 57 |
| 27 | Female | 74 | E | - | - | - | + | ANGC | alive | 65 |
| 28 | Female | 51 | E | - | - | - | + | ANGC | alive | 51 |
| 29 | Male | 57 | N | - | - | - | + | ANGC | alive | 51 |
| 30 | Female | 32 | E | - | - | - | + | ANGC | alive | 48 |
| 31 | Female | 50 | N | - | - | - | + | ANGC | alive | 69 |
| 32 | Female | 35 | E | - | - | - | + | ANGC | alive | 36 |
| 33 | Female | 30 | N | - | - | - | + | ANGC | alive | 33 |
| 34 | Female | 70 | E | - | - | + | + | ANGC | alive | 31 |
| 35 | Male | 48 | N | - | - | + | + | ANGC | alive | 16 |
| 36 | Male | 61 | E | - | - | - | + | ANGC | alive | 25 |
| 37 | Female | 49 | N | - | - | - | + | ANGC | alive | 22 |
| 38 | Female | 49 | E | - | - | - | + | ANGC | alive | 20 |
| 39 | Male | 51 | E | - | - | - | + | ANGC | alive | 12 |
| 40 | Male | 50 | N | - | - | + | + | ANGC | alive | 12 |
| 41 | Male | 68 | E | - | - | + | + | ANGC | alive | 124 |
| 42 | Male | 45 | N | - | - | - | + | ANGC | alive | 117 |
| 43 | Male | 46 | N | - | - | - | + | ANGC | dead | 20 |
| 44 | Male | 69 | E | - | - | - | + | ANGC | dead | 4 |
| 45 | Male | 68 | E | - | - | - | + | ANGC | dead | 11 |

| Sample No. | Gender | Age | Location | CD10 | Bcl-6 | CD138 | MUM1 | DLBCL Subtype | Survival | Duration |
|---|---|---|---|---|---|---|---|---|---|---|
| 46 | Female | 49 | E | - | - | + | + | ANGC | dead | 11 |
| 47 | Female | 77 | E | - | - | - | + | ANGC | dead | 0 |
| 48 | Female | 71 | N | - | - | - | + | ANGC | dead | 4 |
| 49 | Male | 61 | E | - | - | - | + | ANGC | dead | 5 |
| 50 | Male | 72 | E | - | - | - | + | ANGC | dead | 5 |
| 51 | Female | 80 | N | - | - | - | + | ANGC | dead | 14 |
| 52 | Female | 56 | E | - | - | - | + | ANGC | dead | 5 |
| 53 | Male | 40 | E | - | - | - | + | ANGC | dead | 15 |
| 54 | Male | 68 | E | - | - | - | + | ANGC | dead | 4 |
| 55 | Male | 77 | E | - | - | - | + | ANGC | dead | 8 |
| 56 | Male | 70 | N | - | - | + | + | ANGC | dead | 75 |
| 57 | Male | 82 | N | - | - | - | + | ANGC | dead | 32 |
| 58 | Male | 32 | E | - | - | - | - | UC | alive | 50 |

[a] Site of the primary disease: E = extranodal, N = nodal.
[b] DLBCL subtypes: GC = germinal center, AGC = activated germinal center, ANGC = activated non-germinal center, UC = unclassified
[c] duration in months calculated from the date of diagnosis

**Table 4.18** Univariate Cox Proportional Hazard Regression Analysis

| Variable | p-value | Relative Risk | 95% Confidence Interval for Relative Risk | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| GC-DLBCL (vs AGC-, ANGC-, and UC-DLBCL) | 0.483 | 0.790 | 0.410 | 1.524 |
| IL-10 -1087 AA (vs AG and GG) | 0.247 | 1.693 | 0.694 | 4.130 |
| IL-10 -824 TT (vs TC and CC) | 0.858 | 1.063 | 0.545 | 2.071 |
| IL-10 -597 AA (vs AC and CC) | 0.411 | 0.766 | 0.406 | 1.445 |
| CD10 negative (vs positive) | 0.131 | 0.214 | 0.029 | 1.586 |
| Bcl-6 negative (vs positive) | 0.807 | 1.105 | 0.496 | 2.464 |
| CD138 negative (vs positive) | 0.329 | 0.584 | 0.199 | 1.718 |
| MUM1 negative (vs positive) | 0.886 | 0.899 | 0.210 | 3.851 |
| Location Nodal (vs Extra-nodal) | 0.357 | 1.508 | 0.629 | 3.619 |

**Table 4.19** Univariate Cox Proportional Hazard Regression Analysis with CD10

| Variable | p-value | Relative Risk | 95% Confidence Interval for Relative Risk | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| GC-DLBCL (vs AGC-, ANGC-, and UC-DLBCL) | 0.127 | 0.617 | 0.332 | 1.148 |
| IL-10 -1087 AA (vs AG and GG) | 0.321 | 1.576 | 0.642 | 3.869 |
| IL-10 -824 TT (vs TC and CC) | 0.882 | 1.054 | 0.525 | 2.117 |
| IL-10 -597 AA (vs AC and CC) | 0.380 | 0.749 | 0.392 | 1.430 |
| Bcl-6 negative (vs positive) | 0.270 | 1.589 | 0.698 | 3.618 |
| CD138 negative (vs positive) | 0.381 | 0.618 | 0.210 | 1.817 |
| MUM1 negative (vs positive) | 0.718 | 0.765 | 0.178 | 3.282 |
| Location Nodal (vs Extra-nodal) | 0.150 | 1.913 | 0.792 | 4.622 |

**Table 4.20** Univariate Cox Proportional Hazard Regression Analysis with Bcl-6

| Variable | p-value | Relative Risk | 95% Confidence Interval for Relative Risk | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| GC-DLBCL (vs AGC-, ANGC-, and UC-DLBCL) | 0.315 | 0.516 | 0.142 | 1.878 |
| IL-10 -1087 AA (vs AG and GG) | 0.259 | 1.683 | 0.682 | 4.152 |
| IL-10 -824 TT (vs TC and CC) | 0.863 | 1.060 | 0.545 | 2.063 |
| IL-10 -597 AA (vs AC and CC) | 0.394 | 0.759 | 0.403 | 1.431 |
| CD10 negative (vs positive) | 0.078 | 0.157 | 0.020 | 1.234 |
| CD138 negative (vs positive) | 0.312 | 0.567 | 0.189 | 1.701 |
| MUM1 negative (vs positive) | 0.969 | 0.971 | 0.220 | 4.289 |
| Location Nodal (vs Extra-nodal) | 0.361 | 1.529 | 0.615 | 3.802 |

**Table 4.21** Univariate Cox Proportional Hazard Regression Analysis with CD138

| Variable | p-value | Relative Risk | 95% Confidence Interval for Relative Risk | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| GC-DLBCL (vs AGC-, ANGC-, and UC-DLBCL) | 0.527 | 0.809 | 0.421 | 1.558 |
| IL-10 -1087 AA (vs AG and GG) | 0.260 | 1.647 | 0.691 | 3.923 |
| IL-10 -824 TT (vs TC and CC) | 0.938 | 1.027 | 0.517 | 2.042 |
| IL-10 -597 AA (vs AC and CC) | 0.424 | 0.770 | 0.407 | 1.460 |
| CD10 negative (vs positive) | 0.145 | 0.225 | 0.030 | 1.670 |
| Bcl-6 negative (vs positive) | 0.848 | 1.083 | 0.480 | 2.445 |
| MUM1 negative (vs positive) | 0.822 | 0.846 | 0.197 | 3.632 |
| Location Nodal (vs Extra-nodal) | 0.452 | 1.400 | 0.582 | 3.368 |

**Table 4.22** Univariate Cox Proportional Hazard Regression Analysis with MUM1

| Variable | p-value | Relative Risk | 95% Confidence Interval for Relative Risk | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| GC-DLBCL (vs AGC-, ANGC-, and UC-DLBCL) | 0.559 | 0.814 | 0.409 | 1.622 |
| IL-10 -1087 AA (vs AG and GG) | 0.262 | 1.669 | 0.682 | 4.085 |
| IL-10 -824 TT (vs TC and CC) | 0.875 | 1.056 | 0.536 | 2.079 |
| IL-10 -597 AA (vs AC and CC) | 0.396 | 0.757 | 0.398 | 1.439 |
| CD10 negative (vs positive) | 0.132 | 0.214 | 0.029 | 1.589 |
| Bcl-6 negative (vs positive) | 0.850 | 1.082 | 0.478 | 2.452 |
| CD138 negative (vs positive) | 0.344 | 0.594 | 0.202 | 1.748 |
| Location Nodal (vs Extra-nodal) | 0.349 | 1.530 | 0.628 | 3.725 |

# Chapter 5: Discussion

## 5.1 SNP for low IL-10 production in Hong Kong population

In the present study, the predominant allelic frequencies in the Chinese population of Hong Kong for IL-10 $_{-1087}$, IL-10 $_{-824}$ and IL-10 $_{-597}$ were A-allele (97.48%), T-allele (70.14%) and A-allele (70.14%), respectively. The allelic frequencies of all three positions and the frequencies of halpotypes were consistent with earlier reports (Mok et al, 1998). Four haplotypes were observed, ATA (70.49%), ACC (26.04%), GCC (3.13%) and GTA (0.35%).

The allelic frequencies, genotype and haplotypes of SNPs in the IL-10 promoter varies in different ethnical population (Reynard et al, 2000). For Caucasian from UK, the allelic frequencies of IL-10 $_{-1087A}$ and IL-10 $_{-1087G}$ were 51.00% and 49.00% respectively (Reynard et al, 2000). Both A and G allele have similar frequency within the UK population. However, in the Oriental population, the allelic frequencies of IL-10 $_{-1087A}$ and IL-10 $_{-1087G}$ were 94.00% and 6.00%, respectively (Mok et al, 1998). The A-allele is predominant within the Oriental population. In Caucasians, the predominant alleles of the positions of IL-10 $_{-824}$ and IL-10 $_{-597}$ are both C-allele (77.00%) (Reynard et al, 2000). In the Oriental population, the predominant allele at IL-10 $_{-824}$ and IL-10 $_{-597}$ are T-allele (67.00%) and A-allele (67.00%), respectively (Mok et al, 1998). Ethnical difference is also observed in halpotypes of the IL-10 promoter. In Caucasians, all three SNP positions are grouped together forming three haplotypes GCC (49.40%), ACC (28.80%) and ATA (21.80%) (Reynard et al, 2000). In the Orientals, four haplotypes are formed,

they are GCC (2.00%), ACC (30.00%), ATA (64.00%) and GTA (4.00%) (Mok et al, 1998). The haplotype GTA is only observed in the Oriental population but not in the Caucasians. The most important functional implications of these studies were that the genotypes and haplotypes of the IL-10 promoter in Caucasians are responsible for high IL-10 production. In contrast, the genotypes and haloptypes of the IL-10 promoters in the Chinese population are those of low IL-10 production

The SNPs in the human IL-10 promoter affect nuclear factor binding properties and influence the IL-10 gene transcription in vitro (Rees, et al, 2002). The IL-10 base transition at position –1087 lies within a putative ETS transcription factor-binding site (Kube et al, 1995), however, DNA sequence around -1087 position does not correspond to any published ETS consensus binding sites (Wasylyk et al, 1993; Janknecht et al, 1993; Crepieux et al, 1994). It was demonstrated that -1087 A-allele has higher affinity to ETS transcription factor than that of -1087 G-allele. It was found that the actual ETS transcription factor is PU.1, which inhibits the gene expression of IL-10 (Reuss et al, 2002). It has also been demonstrated by the reporter gene assays that particular SNP alleles have variable activities (Crawley et al, 1999). Individuals homozygous for haplotype ATA produce significantly less IL-10 than those without the ATA haplotype. Moreover, in the luciferase reporter assays using the truncated version of IL-10 promoter, significantly higher transcriptional activity was observed using the GCC IL-10 haplotype construct, compared to that of the ACC and ATA constructs (Crawley et al, 1999).

The present study of the SNPs of the IL-10 promoters confirmed the alleles, genotypes and haplotypes in the local Chinese population were those responsible for low

IL-10 production. The microenvironment in which lymphoma arises created with such a background of IL-10 concentration may be different from that in Caucasian population. The significance of SNP of the IL-10 promoter in other types of NHL requires further investigation.

## 5.2 NHL in low IL-10 production population

### 5.2.1 The relationship between IL-10 and NHL

It has been demonstrated that the serum IL-10 level is elevated in NHL patient, and this elevated serum IL-10 level is related to poor prognosis (Blay et al, 1993). The biological significance of the unfavorable prognostic value of IL-10 remains unclear. It was hypothesized that this unfavorable prognosis is related to the immunosuppressive properties of IL-10. IL-10 inhibits the antigen-dependent T-cell proliferation by downregulating the expression of MHC class II on monocytes and macrophages in vitro. Therefore, the high serum concentration of IL-10 in NHL patients and, possibly, in the microenvironment of the tumor site may exert an inhibitory effect on macrophages and antigen-specific T-cell response at tumor site. These may contribute to the lymphoma progression in vivo.

### 5.2.2 Allelic frequencies and haplotype of the IL-10 promoter in NHL

In 144 NHL patients of our hospital, the predominant allelic frequencies of IL-10 $_{-1087}$, IL-10 $_{-824}$ and IL-10 $_{-597}$ were A-allele (96.53%), T-allele (73.38%) and A-allele (73.38%), respectively. Four haplotypes were also observed: ATA (70.49%), ACC (26.04%), GCC (3.13%) and GTA (0.35%). We found no significant differences between

the NHL patients and the normal control group in allelic frequencies, genotypes and haplotypes. Hence, we have no evidence suggesting that the NHL in our locality is associated with genetic predispositions of a high serum IL-10. In this retrospective study, sera of the NHL patients were not available for the assessment of IL-10 levels. If serum IL-10 did elevate in our NHL patients, as shown in other studies, the main determinant of the serum IL-10 might then not be related to the SNPs of the IL-10 promoter.

A diverged spectrum of lymphomas arising from different cells of origin is classified under the term "NHL", a equally diverged spectrum of clinical behaviors and biochemical parameters are to be expected. Further studies should try to focus on the correlation of SNP of the IL-10 promoters in each specific NHL. A direct assessment of the serum levels of IL-10 at different stages of the diseases will be helpful in clarifying the role of IL-10 in NHL.

### 5.3 Classification of DLBCL

#### 5.3.1 Current prognostic analysis

The International Prognosis Index (IPI) is currently the standard approach to assess prognosis in DLBCL (Shipp et al, 1993). However, identical IPI groups include patients with heterogeneous prognoses, such as low-intermediate and high-intermediate categories, with a long term survival of 40-50% (Pedersen et al, 2004). Therefore, novel biomarkers might be incorporated into current risk assessment model of prognosis. Subtypes of DLBCL with prognostic significance can be identified by the patterns of gene expression using DNA microarray analyses or by the patterns of antigen expression using immunohistochemical analyses. The latter is a more practical and economical

classification strategy of the disease in routine clinical practice.

### 5.3.2 DLBCL subtypes distribution in Hong Kong is different from Caucasian

In the immunohistochemical analyses of DLBCL, we observed differences in the spectrum of subtypes of DLBCL in our locality. The overall frequencies of expression for CD10, Bcl-6, CD138 and MUM1/IRF4 were as follows: 23 (21.30%), 41 (37.96%), 25 (23.15%) and 94 (84.04%), respectively. When compared to the frequency of expression in Chang's studies by Chi-square test, significant differences were observed in all 4 antigens: CD10 ($p = 0.035$, OR = 0.440, 95% CI: 0.203-0.954), CD138 ($p = 0.034$, OR = 3.715, 95% CI: 1.055-13.079) and MUM1/IRF4 ($p < 0.001$, OR = 6.104, 95% CI: 2.673-13.939).

The frequencies of each subtype of DLBCL were as follows: 9 (8.33%) GC-DLBCL, 28 (25.93%) AGC-DLBCL, 60 (55.56%) ANGC-DLBCL and the remaining 11 cases (10.19%) were unclassified. Significant differences were also observed for the frequency of each subtype of DLBCL in Chang's studies by Chi-square test: GC-DLBCL ($p < 0.001$, OR = 0.157, 95% CI: 0.061-0.404) and ANGC-DLBCL ($p = 0.001$, OR = 3.980, 95% CI: 1.767-8.965).

The above observation suggested that there were ethnical differences in the subtypes of DLBCL. ANGC-DLBCL had higher frequency in the local population, and the most common subtypes in Chang's series, GC-DLBCL, was rare.

### 5.4 IL-10 and DLBCL

The relationship between IL-10 and DLBCL remains contradictory. IL-10 is an important immuno-regulatory cytokine produced by healthy as well as neoplastic B-cells, acting as an autocrine growth factor. IL-10 plays an important role in controlling the balance between cellular and humoral immune system. IL-10 is also a strong immunosuppressive cytokine that inhibits the proimflammatory T-cells (Moore et al, 2001; Benjamin et al, 1992; Rousset et al, 1992 and Levy and Brouet, 1994). Therefore, it was hypothesized that IL-10 may adversely affect the prognosis and clinical outcomes of DLBCL patients (Lech-Maranda et al, 2004). Earlier report did not support a prognosis correlation between IL-10 and DLBCL (Cortes et al, 1995). Two further reports show contrasting results of IL-10 and outcomes of DLBCL (Lech-Maranda et al, 2004 and Berglund et al, 2005), and hence it is hypothesized that increased serum levels of IL-10 reflect an enhanced activation of the immune system on more aggressive disease, but its potential action as a growth factor for lymphoma cells or as a suppressor of macrophages or T-cell functions (Voorzanger et al, 1996).

In this study, there were no significant differences between DLBCL patients and normal control in allelic frequencies. Significant differences were, however, observed in genotype IL-$10_{-824TT}$ ($p = 0.019$, OR = 0.541, 95% CI: 0.324-0.906) and IL-$10_{-824TC}$ ($p = 0.003$, OR = 2.187, 95% CI: 1.310-3.651). When comparing IL-$10_{-824CC}$, there was no significant difference found, suggesting that only the T-allele, which is responsible for the low IL-10 secretion (Lech-Maranda et al, 2004), was associated with DLBCL. This association of low IL-10 promoter allele has been observed by others in NHL. The relative lack of IL-10 might alter the immune responses and might allow the lymphoma

arise or progress under the influence of other pro-lymphoma cytokines (Cunningham et al, 2003).

### 5.5 SNP of IL-10 promoter in DLBCL subtypes

#### 5.5.1 Allelic frequencies and haplotype of DLBCL subtypes

In three subtypes of DLBCL, the allelic frequencies of all the cases were having no significant differences from the normal control. However, in GC-DLBCL and ANGC-DLBCL, at IL-10 $_{-824}$ significant differences were observed in TT and TC genotypes in GC-DLBCL, whereas TC genotype in ANGC-DLBCL. This suggested that the low production T-allele in IL-10 promoter was associated with GC- and ANGC-DLBCL patients..

#### 5.5.2 Rare haplotypes were discovered in DLBCL

Besides those typical haplotypes, rare haplotypes, ACA and ATC, were observed in the local DLBCL patients. These haplotypes were not observed in normal control. The haplotype frequency of ACA in DLBCL was significantly different from the control group ($p < 0.001$, OR = 0.944, 95% CI: 0.914-0.975). These two new haplotypes were found in the Chinese but not in the Caucasians (Hu et al, 2003). The relationship between the haplotype ACA and IL-10 production, however, has not been studied. Further investigation, such as luciferase reporter assay and ELISA in these haplotypes, is needed to clarify the relationship between these haplotypes and the secretion of IL-10. Whether these haplotypes predisposed to the development of DLBCL or the variations of clinical behaviors of the local DLBCL remains to be resolved.

### 5.6 Overall survival Analysis

Previous studies revealed that CD10 and Bcl-6 are the markers that are strongly associated with the germinal center B-cell (Lossos et al, 2001; Ohshima et al, 2001, Uherova et al, 2001 and Barrans et al, 2002). The expression of CD10 and/or Bcl-6 in DLBCL is associated with better prognosis. Recent study using panels of GC-B-cell and activation markers by immunohistochemistry on paraffin-embedded tissues developed these previous observations on DLBCL further by suggesting three distinguishable expression patterns corresponding to GC-B-cell, AGC-B-cell and ANG-B-cell. These expression patterns appear to represent distinct clinicopathologic subtypes of DLBCLs that have prognostic significances (Chang et al, 2004).

#### 5.6.1 Univariate Cox Proportional Hazards Regression Analysis

Univariate Cox Proportional Hazards Regression Analysis was performed in both SNP positions of the IL-10 promoter and the biomarkers of all de novo DLBCL subtype, so that the impact of both SNP and biomarkers on the overall survival of DLBCL patients can be assessed. In our data, both SNP positions and DLBCL biomarkers had no impact on the overall survival of the DLBCL patients. These results were different from previous report that DLBCL subtypes, CD10 and MUM1 have influences on the overall survival of comparable numbers of DLBCL patients (Chang et al, 2004). The different distribution of DLBCL subtypes in our locality and the low IL-10 production SNP haplotypes background in the Hong Kong population may account for these differences. Subtyping of the DLBCL and the IL-10 promoter SNP might not have similar clinical applications as in the Caucasion population.

### 5.6.2 Bivariate Cox Proportional Hazards Regression Analysis

In order to investigate the effect of each DLBCL biomarkers in each prognosis parameters on the impact of the overall survival of DLBCL patients, Bivariate Cox Proportional Hazards Regression Analysis was performed. In our data, we could not see any significant impact on each prognosis parameters together with each DLBCL biomarkers. We could not detect any associations of CD10 and Bcl-6 with prognosis of DLBCL patients. These contrast to the reports of better prognosis of DLBCL with the expressions of CD10 and Bcl-6 detected by others. Moreover, no significant impact was obtained with the prognosis analysis in all SNP positions of the IL-10 promoter. Hence, genotyping of IL-10 promoter and subtyping of DLBCL do not have any prognostic significance in the management of the local DLBCL patients.

# Chapter 6: Conclusion

The SNP genotypings of IL-10 promoter in positions -597, -824 and -1087 were performed in normal health control and NHL patients. Detailed analyses of SNP genotypings of the IL-10 promoter in different subtypes of DLBCL patients were also performed.

In normal control, NHL and DLBCL patients, the most common alleles at positions -597, -824 and -1087 were A-allele, T-allele and A-allele, respectively. The most common genotypes at positions -597, -824 and -1087 in normal control and NHL patients were homozygous AA, homozygous TT and homozygous AA, respectively. The most common haplotype in all categories were ATA. The SNP genotypes of Hong Kong population were related to low IL-10 production. It is, therefore, likely that both NHL, including DLBCL, were developed in low IL-10 production backgrounds.

The genotype of SNP of the IL-10 promoter in the control population at position -597 is linked with -824. The A-allele at -597 was followed by T-allele at -824; and C-allele at -597 was followed by C-allele at -824. In DLBCL patients, the most common genotype at -824 was heterozygous TC instead of homozygous TT as in normal controls. In some DLBCL patients, this linkage between positions -597 and -824 was disrupted. Rare haplotypes, ACA ATC and GCA, were discovered in DLBCL. These rare haplotypes were not observed in normal control and NHL patients. These rare haplotypes might be associated with the susceptibility of DLBCL in the local population.

The most common subtype of DLBCL in our series was ANGC-DLBCL, in

contrast to the most common subtype of GC-DLBCL in the Caucasian population.. The overall distribution of DLBCL subtypes in our locality was also different from those in the Caucasian population. These indicated that the spectrum of DLBCL subtypes may be different among different ethnical groups.

Prognostic analyses of the genotypes of each SNP positions and DLBCL biomarkers were performed. There were no significant impacts on the overall survival with each of the parameters analyzed individually and in combinations. These indicated that the SNP genotypes of the IL-10 promoter and the DLBCL subtyping have little, if any, prognostic value in the local DLBCL patients.

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